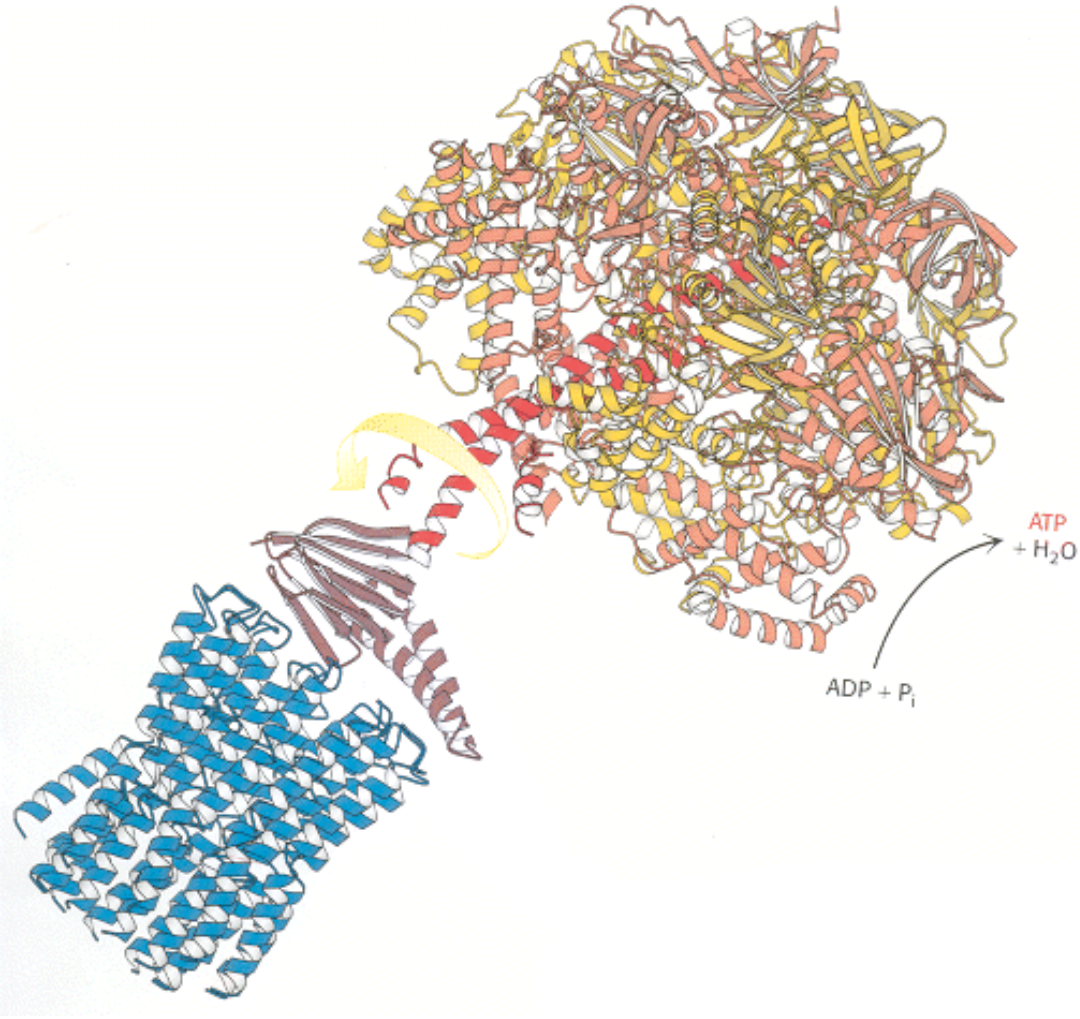
II. Transducing and Storing Energy



ATP synthase. This enzyme is a molecular assembly that transduces the free energy associated with a proton gradient to the chemical energy associated with ATP. The proton gradient drives the rotation of one component of the assembly within the other. This rotational motion in turn drives the synthesis and release of ATP.

14. Metabolism: Basic Concepts and Design

The concepts of conformation and dynamics developed in Part I—especially those dealing with the specificity and catalytic power of enzymes, the regulation of their catalytic activity, and the transport of molecules and ions across membranes—enable us to now ask questions fundamental to biochemistry:

1. *How does a cell extract energy and reducing power from its environment?*

2. *How does a cell synthesize the building blocks of its macromolecules and then the macromolecules themselves?*

These processes are carried out by a highly integrated network of chemical reactions that are collectively known as *metabolism*.

More than a thousand chemical reactions take place in even as simple an organism as *Escherichia coli*. The array of reactions may seem overwhelming at first glance. However, closer scrutiny reveals that metabolism has a *coherent design containing many common motifs*. These motifs include the use of an energy currency and the repeated appearance of a limited number of activated intermediates. In fact, a group of about 100 molecules play central roles in all forms of life. Furthermore, although the number of reactions in metabolism is large, the number of *kinds* of reactions is small and the mechanisms of these reactions are usually quite simple. Metabolic pathways are also regulated in common ways. The purpose of this chapter is to introduce some general principles and motifs of metabolism to provide a foundation for the more detailed studies to follow.

14.0.1. Cells Transform Different Types of Energy

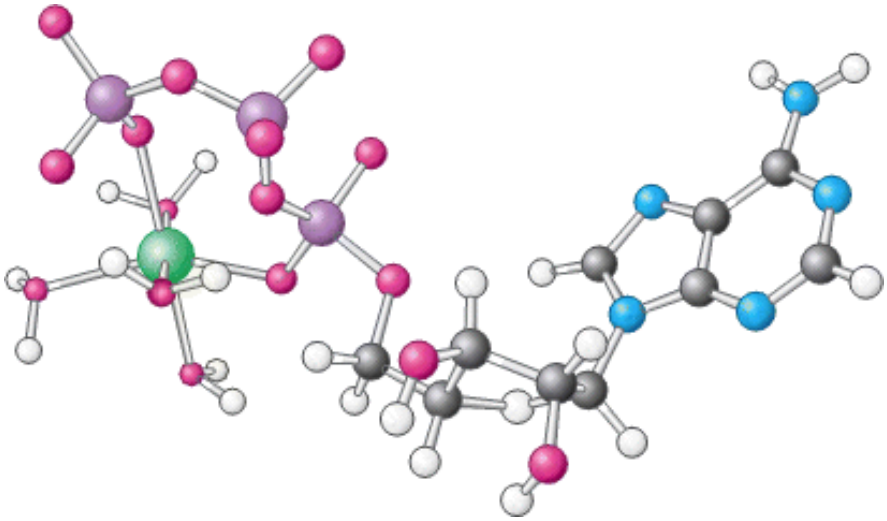
Living organisms require a continual input of free energy for three major purposes: (1) the performance of mechanical work in muscle contraction and other cellular movements, (2) the active transport of molecules and ions, and (3) the synthesis of macromolecules and other biomolecules from simple precursors. The free energy used in these processes, which maintain an organism in a state that is far from equilibrium, is derived from the environment.

The First Law of Thermodynamics states that energy can be neither created nor destroyed. The amount of energy in the universe is constant. Nevertheless, energy can be converted from one form into another.

Photosynthetic organisms, or *phototrophs*, use the energy of sunlight to convert simple energy-poor molecules into more-complex energy-rich molecules that serve as fuels. In other words, photosynthetic organisms transform light energy into chemical energy. Indeed, this transformation is ultimately the primary source of chemical energy for the vast majority of organisms, human beings included. *Chemotrophs*, which include animals, obtain chemical energy through the oxidation of foodstuffs generated by phototrophs.

Chemical energy obtained from the oxidation of carbon compounds may be transformed into the unequal distribution of ions across a membrane, resulting in an ion gradient. This gradient, in turn, is an energy source that can be used to move molecules across membranes, that can be converted into yet other types of chemical energy, or that can convey information in the form of nerve impulses. In addition, chemical energy can be transduced into mechanical energy. We convert the chemical energy of a fuel into structural alterations of contractile proteins that result in muscle contraction and movement. Finally, chemical energy powers the reactions that result in the synthesis of biomolecules.

At any given instant in a cell, thousands of energy transformations are taking place. Energy is being extracted from fuels and used to power biosynthetic processes. These transformations are referred to as *metabolism* or *intermediary metabolism*.



Hummingbirds are capable of prodigious feats of endurance. For instance, the tiny ruby-throated hummingbird can store enough fuel to fly across the Gulf of Mexico, a distance of some 500 miles, without resting. This achievement is possible because of the ability to convert fuels into the cellular energy currency, ATP, represented by the model at the right. [(Left) K. D. McGraw/Rainbow.]

14.1. Metabolism Is Composed of Many Coupled, Interconnecting Reactions

Metabolism is essentially a linked series of chemical reactions that begins with a particular molecule and converts it into some other molecule or molecules in a carefully defined fashion (Figure 14.1). There are many such defined pathways in the cell (Figure 14.2), and we will examine a few of them in some detail later. These pathways are interdependent, and their activity is coordinated by exquisitely sensitive means of communication in which allosteric enzymes are predominant (Section 10.1). We will consider the principles of this communication in Chapter 15.

We can divide metabolic pathways into two broad classes: (1) those that convert energy into biologically useful forms and (2) those that require inputs of energy to proceed. Although this division is often imprecise, it is nonetheless a useful distinction in an examination of metabolism. Those reactions that transform fuels into cellular energy are called *catabolic reactions* or, more generally, *catabolism*.

$$\text{Fuels (carbohydrates, fats)} \xrightarrow{\text{Catabolism}} CO_2 + H_2O + \text{useful energy}$$

Those reactions that require energy—such as the synthesis of glucose, fats, or DNA—are called *anabolic reactions* or *anabolism*. The useful forms of energy that are produced in catabolism are employed in anabolism to generate complex structures from simple ones, or energy-rich states from energy-poor ones.

$$\text{Useful energy} + \text{small molecules} \xrightarrow{\text{Anabolism}} \text{complex molecules}$$

Some pathways can be either anabolic or catabolic, depending on the energy conditions in the cell. They are referred to as *amphibolic pathways*.

14.1.1. A Thermodynamically Unfavorable Reaction Can Be Driven by a Favorable Reaction

Conceptual Insights, Energetic Coupling, offers a graphical presentation of how enzymatic coupling enables a favorable reaction to drive an unfavorable reaction.

How are specific pathways constructed from individual reactions? A pathway must satisfy minimally two criteria: (1) the individual reactions must be *specific* and (2) the entire set of reactions that constitute the pathway must be *thermodynamically favored*. A reaction that is specific will yield only one particular product or set of products from its reactants. As discussed in Chapter 8, a function of enzymes is to provide this specificity. The thermodynamics of metabolism is most readily approached in terms of free energy, which was discussed in Sections 1.3.3, 8.2.1, and 8.2.2. A reaction can occur spontaneously only if ΔG, the change in free energy, is negative. Recall that ΔG for the formation of products C and D from substrates A and B is given by

$$\Delta G = \Delta G^{\circ\prime} + RT \ln \frac{[C][D]}{[A][B]}$$

Thus, the ΔG of a reaction depends on the *nature* of the reactant and products (expressed by the $\Delta G^{\circ\prime}$ term, the standard free-energy change) and on their *concentrations* (expressed by the second term).

An important thermodynamic fact is that *the overall free-energy change for a chemically coupled series of reactions is equal to the sum of the freeenergy changes of the individual steps*. Consider the following reactions:

$$\begin{array}{ll} A \rightleftharpoons B + C & \Delta G^{\circ\prime} = +5 \text{ kcal mol}^{-1} (+21 \text{ kJ mol}^{-1}) \\ B \rightleftharpoons D & \Delta G^{\circ\prime} = -8 \text{ kcal mol}^{-1} (-34 \text{ kJ mol}^{-1}) \\ \hline A \rightleftharpoons C + D & \Delta G^{\circ\prime} = -3 \text{ kcal mol}^{-1} (-13 \text{ kJ mol}^{-1}) \end{array}$$

Under standard conditions, A cannot be spontaneously converted into B and C, because ΔG is positive. However, the conversion of B into D under standard conditions is thermodynamically feasible. Because free- energy changes are additive, the conversion of A into C and D has a $\Delta G^{\circ\prime}$ of -3 kcal mol^{-1} (-13 kJ mol^{-1}), which means that it can occur spontaneously under standard conditions. Thus, *a thermodynamically unfavorable reaction can be driven by a thermodynamically favorable reaction to which it is coupled*. In this example, the chemical intermediate B, common to both reactions, couples the reactions. Thus, metabolic pathways are formed by the coupling of enzyme-catalyzed reactions such that the overall free energy of the pathway is negative.

14.1.2. ATP Is the Universal Currency of Free Energy in Biological Systems

Just as commerce is facilitated by the use of a common currency, the commerce of the cell—metabolism—is facilitated by the use of a common energy currency, *adenosine triphosphate* (ATP). Part of the free energy derived from the oxidation of foodstuffs and from light is transformed into this highly accessible molecule, which acts as the free-energy donor in most energy-requiring processes such as motion, active transport, or biosynthesis.

ATP is a nucleotide consisting of an adenine, a ribose, and a triphosphate unit (Figure 14.3). The active form of ATP is usually a complex of ATP with Mg^{2+} or Mn^{2+} (Section 9.4.2). In considering the role of ATP as an energy carrier, we can focus on its triphosphate moiety. *ATP is an energy-rich molecule because its triphosphate unit contains two phosphoanhydride bonds*. A large amount of free energy is liberated when ATP is hydrolyzed to adenosine diphosphate (ADP) and orthophosphate (P_i) or when ATP is hydrolyzed to adenosine monophosphate (AMP) and pyrophosphate (PP_i).

$$\text{ATP} + \text{H}_2\text{O} \rightleftharpoons \text{AMP} + \text{PP}_\text{i} \qquad \Delta G^{\circ\prime} = -10.9 \text{ kcal mol}^{-1} (-45.6 \text{ kJ mol}^{-1})$$

$$\text{ATP} + \text{H}_2\text{O} \rightleftharpoons \text{ADP} + \text{P}_\text{i} \qquad \Delta G^{\circ\prime} = -7.3 \text{ kcal mol}^{-1} (-30.5 \text{ kJ mol}^{-1})$$

The precise $\Delta G^{\circ\prime}$ for these reactions depends on the ionic strength of the medium and on the concentrations of Mg^{2+} and other metal ions. Under typical cellular concentrations, the actual ΔG for these hydrolyses is approximately -12 kcal mol^{-1} (-50 kJ mol^{-1}).

The free energy liberated in the hydrolysis of ATP is harnessed to drive reactions that require an input of free energy, such as muscle contraction. In turn, ATP is formed from ADP and P_i when fuel molecules are oxidized in chemotrophs or when light is trapped by phototrophs. *This ATP—ADP cycle is the fundamental mode of energy exchange in biological systems.*

Some biosynthetic reactions are driven by hydrolysis of nucleoside triphosphates that are analogous to ATP—namely, guanosine triphosphate (GTP), uridine triphosphate (UTP), and cytidine triphosphate (CTP). The diphosphate forms of these nucleotides are denoted by GDP, UDP, and CDP, and the monophosphate forms by GMP, UMP, and CMP. Enzymes can catalyze the transfer of the terminal phosphoryl group from one nucleotide to another. The phosphorylation of nucleoside monophosphates is catalyzed by a family of nucleoside monophosphate kinases, as discussed in Section 9.4.1. The phosphorylation of nucleoside diphosphates is catalyzed by nucleoside diphosphate kinase, an enzyme with broad specificity. It is intriguing to note that, although all of the nucleotide triphosphates are energetically equivalent, ATP is nonetheless the primary cellular energy carrier. In addition, two important electron carriers, NAD^+ and FAD, are derivatives of ATP. *The role of ATP in energy metabolism is paramount.*

14.1.3. ATP Hydrolysis Drives Metabolism by Shifting the Equilibrium of Coupled Reactions

How does coupling to ATP hydrolysis make possible an otherwise unfavorable reaction? Consider a chemical reaction that is thermodynamically unfavorable without an input of free energy, a situation common to many biosynthetic reactions. Suppose that the standard free energy of the conversion of compound A into compound B is +4.0 kcal mol^{-1} (+13 kJ mol^{-1}):

$$\text{A} \rightleftharpoons \text{B} \qquad \Delta G^{\circ\prime} = +4.0 \text{ kcal mol}^{-1} (+13 \text{ kJ mol}^{-1})$$

The equilibrium constant K'_{eq} of this reaction at 25°C is related to $\Delta G^{\circ\prime}$ (in units of kilocalories per mole) by

$$K'_{eq} = [\text{B}]_{eq}/[\text{A}]_{eq} = 10^{-\Delta G^{\circ\prime}/1.36} = 1.15 \times 10^{-3}$$

Thus, net conversion of A into B cannot occur when the molar ratio of B to A is equal to or greater than 1.15×10^{-3}. However, A can be converted into B under these conditions if the reaction is coupled to the hydrolysis of ATP. The new overall reaction is

$$\text{A} + \text{ATP} + \text{H}_2\text{O} \rightleftharpoons \text{B} + \text{ADP} + \text{P}_\text{i} + \text{H}^+ \qquad \Delta G^{\circ\prime} = -3.3 \text{ kcal mol}^{-1} (-13.8 \text{ kJ mol}^{-1})$$

Its standard free-energy change of -3.3 kcal mol^{-1} (-13.8 kJ mol^{-1}) is the sum of the value of $\Delta G^{\circ\prime}$ for the conversion of

A into B [+4.0 kcal mol^{-1} (+12.6 kJ mol^{-1})] and the value of $\Delta G^{\circ\prime}$ for the hydrolysis of ATP [-7.3 kcal mol^{-1} (-30.5 kJ mol^{-1})]. At pH 7, the equilibrium constant of this coupled reaction is

$$K'_{eq} = \frac{[B]_{eq}}{[A]_{eq}} \times \frac{[ADP]_{eq}[P_i]_{eq}}{[ATP]_{eq}} = 10^{3.3/1.36} = 2.67 \times 10^2$$

At equilibrium, the ratio of [B] to [A] is given by

$$\frac{[B]_{eq}}{[A]_{eq}} = K'_{eq} \frac{[ATP]_{eq}}{[ADP]_{eq}[P_i]_{eq}}$$

The ATP-generating system of cells maintains the $[ATP]/[ADP][P_i]$ ratio at a high level, typically of the order of 500 M^{-1}. For this ratio,

$$\frac{[B]_{eq}}{[A]_{eq}} = 2.67 \times 10^2 \times 500 = 1.34 \times 10^5$$

which means that the hydrolysis of ATP enables A to be converted into B until the [B]/[A] ratio reaches a value of 1.34×10^5. This equilibrium ratio is strikingly different from the value of 1.15×10^{-3} for the reaction A $\rightarrow$ B in the absence of ATP hydrolysis. In other words, coupling the hydrolysis of ATP with the conversion of A into B has changed the equilibrium ratio of B to A by a factor of about 10^8.

We see here the thermodynamic essence of ATP's action as an *energy-coupling agent*. Cells maintain a high level of ATP by using oxidizable substrates or light as sources of free energy. The hydrolysis of an ATP molecule in a coupled reaction then changes the equilibrium ratio of products to reactants by a very large factor, of the order of 10^8. More generally, the hydrolysis of *n* ATP molecules changes the equilibrium ratio of a coupled reaction (or sequence of reactions) by a factor of 10^{8n}. For example, the hydrolysis of three ATP molecules in a coupled reaction changes the equilibrium ratio by a factor of 10^{24}. Thus, *a thermodynamically unfavorable reaction sequence can be converted into a favorable one by coupling it to the hydrolysis of a sufficient number of ATP molecules in a new reaction*. It should also be emphasized that A and B in the preceding coupled reaction may be interpreted very generally, not only as different chemical species. For example, A and B may represent activated and unactivated conformations of a protein; in this case, phosphorylation with ATP may be a means of conversion into an activated conformation. Such a conformation can store free energy, which can then be used to drive a thermodynamically unfavorable reaction. Through such changes in conformation, molecular motors such as myosin, kinesin, and dynein convert the chemical energy of ATP into mechanical energy (Chapter 34). Indeed, this conversion is the basis of muscle contraction.

Alternatively, A and B may refer to the concentrations of an ion or molecule on the outside and inside of a cell, as in the active transport of a nutrient. The active transport of Na^+ and K^+ across membranes is driven by the phosphorylation of the sodium-potassium pump by ATP and its subsequent dephosphorylation (Section 13.2.1).

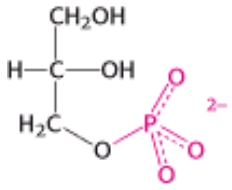
14.1.4. Structural Basis of the High Phosphoryl Transfer Potential of ATP

As illustrated by molecular motors (Chapter 34) and ion pumps (Section 13.2), phosphoryl transfer is a common means of energy coupling. Furthermore, as we shall see in Chapter 15, phosphoryl transfer is also widely used in the intracellular transmission of information. What makes ATP a particularly efficient phosphoryl-group donor? Let us compare the standard free energy of hydrolysis of ATP with that of a phosphate ester, such as glycerol 3-phosphate:

$$\text{ATP} + \text{H}_2\text{O} \rightleftharpoons \text{ADP} + \text{P}_i + \text{H}^+ \qquad \Delta G^{\circ\prime} = -7.3 \text{ kcal mol}^{-1} (-30.5 \text{ kJ mol}^{-1})$$

$$\text{Glycerol 3-phosphate} + \text{H}_2\text{O} \rightleftharpoons \text{glycerol} + \text{P}_i \qquad \Delta G^{\circ\prime} = -2.2 \text{ kcal mol}^{-1} (-9.2 \text{ kJ mol}^{-1})$$

The magnitude of $\Delta G^{\circ\prime}$ for the hydrolysis of glycerol 3-phosphate is much smaller than that of ATP, which means that ATP has a stronger tendency to transfer its terminal phosphoryl group to water than does glycerol 3-phosphate. In other words, ATP has a higher *phosphoryl transfer potential (phosphoryl-group transfer potential)* than does glycerol 3-phosphate.



Glycerol 3-phosphate

What is the structural basis of the high phosphoryl transfer potential of ATP? Because $\Delta G^{\circ\prime}$ depends on the *difference* in free energies of the products and reactants, the structures of both ATP and its hydrolysis products, ADP and P_i, must be examined to answer this question. Three factors are important: *resonance stabilization*, *electrostatic repulsion*, and *stabilization due to hydration*. ADP and, particularly, P_i, have greater resonance stabilization than does ATP. Orthophosphate has a number of resonance forms of similar energy (Figure 14.4), whereas the γ-phosphoryl group of ATP has a smaller number.

Forms like that shown in Figure 14.5 are unfavorable because a positively charged oxygen atom is adjacent to a positively charged phosphorus atom, an electrostatically unfavorable juxtaposition. Furthermore, at pH 7, the triphosphate unit of ATP carries about four negative charges. These charges repel one another because they are in close proximity. The repulsion between them is reduced when ATP is hydrolyzed. Finally, water can bind more effectively to ADP and P_i than it can to the phosphoanhydride part of ATP, stabilizing the ADP and P_i by hydration.

ATP is often called a high-energy phosphate compound, and its phosphoanhydride bonds are referred to as high-energy bonds. Indeed, a "squiggle" (~P) is often used to indicate such a bond. Nonetheless, there is nothing special about the bonds themselves. *They are high-energy bonds in the sense that much free energy is released when they are hydrolyzed*, for the aforegiven reasons.

14.1.5. Phosphoryl Transfer Potential Is an Important Form of Cellular Energy Transformation

The standard free energies of hydrolysis provide a convenient means of comparing the phosphoryl transfer potential of phosphorylated compounds. Such comparisons reveal that ATP is not the only compound with a high phosphoryl transfer potential. In fact, some compounds in biological systems have a higher phosphoryl transfer potential than that of ATP. These compounds include phosphoenolpyruvate (PEP), 1,3-bisphosphoglycerate (1,3-BPG), and creatine phosphate (Figure 14.6). Thus, PEP can transfer its phosphoryl group to ADP to form ATP. Indeed, this is one of the ways in which ATP is generated in the breakdown of sugars (Sections 14.2.1, 16.1.6, and 16.1.7). It is significant that ATP has a phosphoryl transfer potential that is intermediate among the biologically important phosphorylated molecules (Table 14.1). *This intermediate position enables ATP to function efficiently as a carrier of phosphoryl groups.*

Creatine phosphate in vertebrate muscle serves as a reservoir of high-potential phosphoryl groups that can be readily transferred to ATP. Indeed, we use creatine phosphate to regenerate ATP from ADP every time we exercise strenuously.

This reaction is catalyzed by *creatine kinase*.

$$\text{Creatine phosphate} + \text{ADP} + \text{H}^+ \overset{\text{Creatine kinase}}{\rightleftharpoons} \text{ATP} + \text{creatine}$$

At pH 7, the standard free energy of hydrolysis of creatine phosphate is -10.3 kcal mol^{-1} (-43.1 kJ mol^{-1}), compared with -7.3 kcal mol^{-1} (-30.5 kJ mol^{-1}) for ATP. Hence, the standard free-energy change in forming ATP from creatine phosphate is -3.0 kcal mol^{-1} (-12.6 kJ mol^{-1}), which corresponds to an equilibrium constant of 162.

$$K_{eq} = \frac{[\text{ATP}][\text{creatine}]}{[\text{ADP}][\text{creatine phosphate}]} = 10^{-\Delta G^{\circ\prime}/(2.303RT)} = 10^{3/1.36} = 162$$

In resting muscle, typical concentrations of these metabolites are [ATP] = 4 mM, [ADP] = 0.013 mM, [creatine phosphate] = 25 mM, and [creatine] = 13 mM. The amount of ATP in muscle suffices to sustain contractile activity for less than a second. The abundance of creatine phosphate and its high phosphoryl transfer potential relative to that of ATP make it a highly effective phosphoryl buffer. Indeed, creatine phosphate is the major source of phosphoryl groups for ATP regeneration for a runner during the first 4 seconds of a 100-meter sprint. After that, ATP must be generated through metabolism (Figure 14.7).

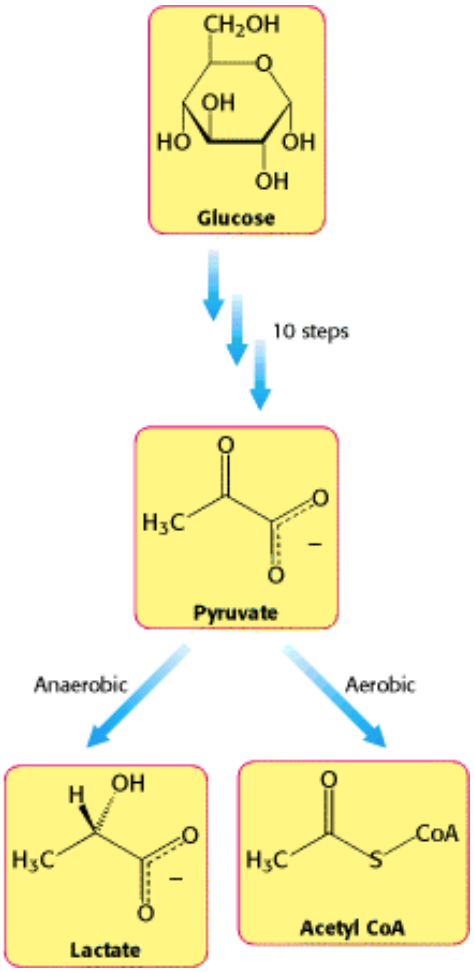


Figure 14.1. Glucose Metabolism. Glucose is metabolized to pyruvate in 10 linked reactions. Under anaerobic conditions, pyruvate is metabolized to lactate and, under aerobic conditions, to acetyl CoA. The glucose-derived carbons are subsequently oxidized to CO_2.

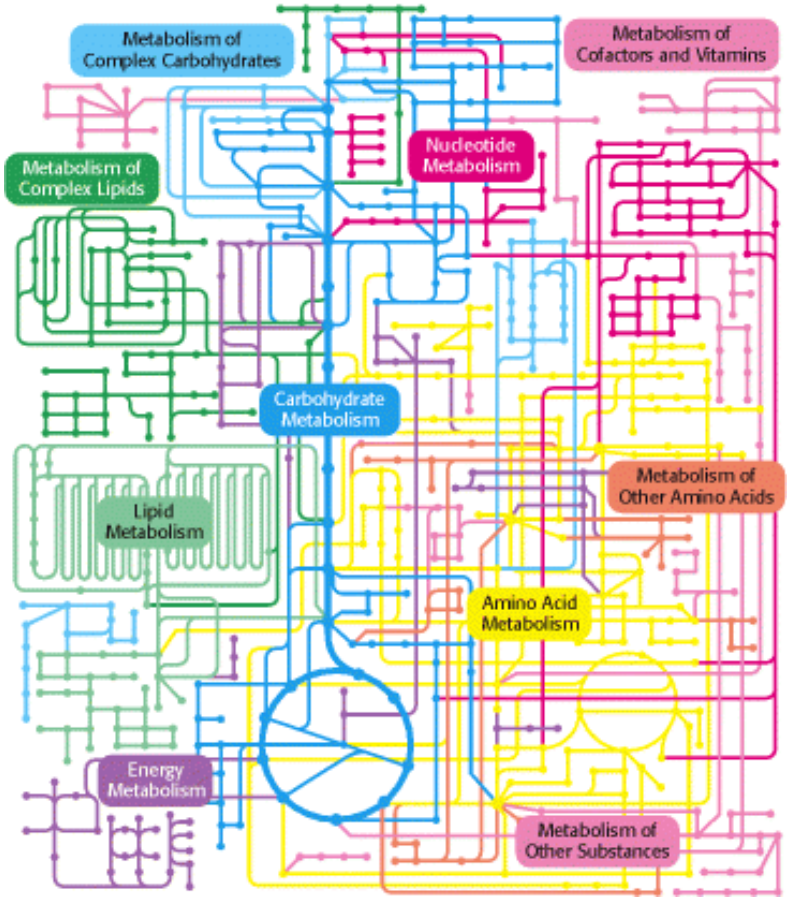


Figure 14.2. Metabolic Pathways. [From the Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg).]

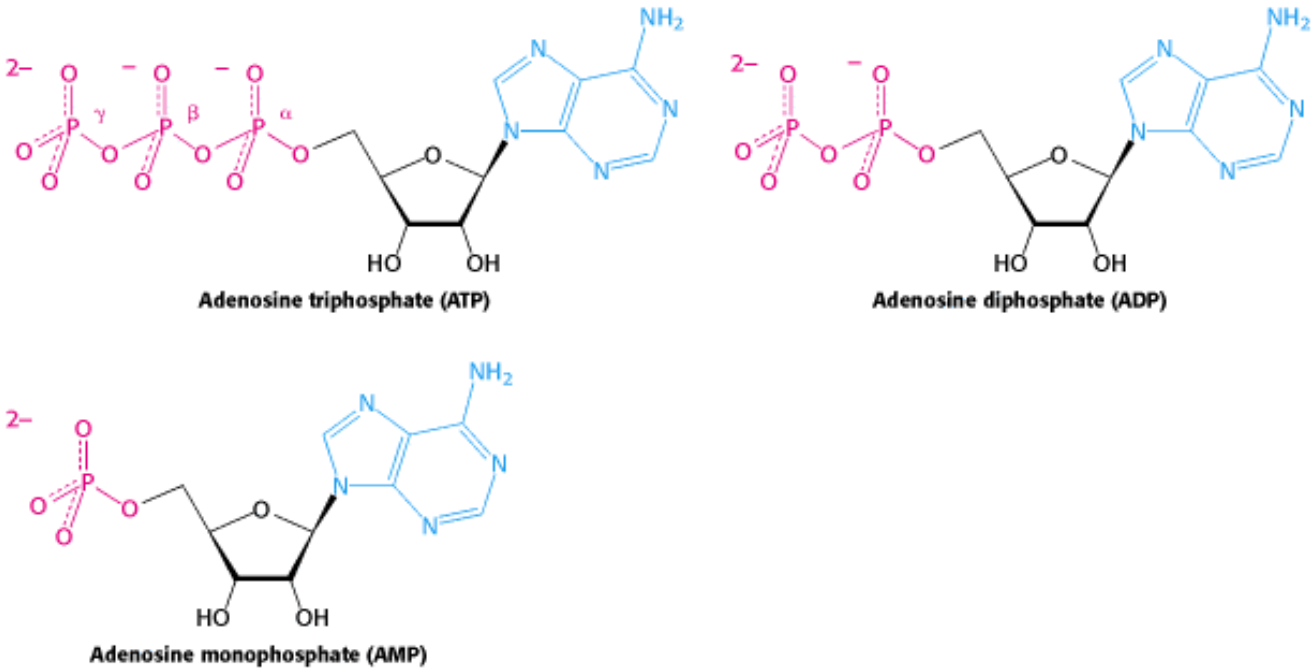


Figure 14.3. Structures of ATP, ADP, and AMP. These adenylates consist of adenine (blue), a ribose (black), and a tri-, di-, or monophosphate unit (red). The innermost phosphorus atom of ATP is designated P_{α}, the middle one P_{β}, and the outermost one P_{γ}.

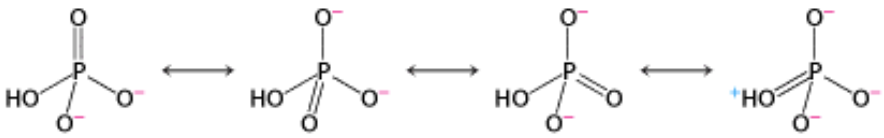


Figure 14.4. Resonance Structures of Orthophosphate.

Figure 14.5. Improbable Resonance Structure. The structure contributes little to the terminal part of ATP, because two positive charges are placed adjacent to each other.

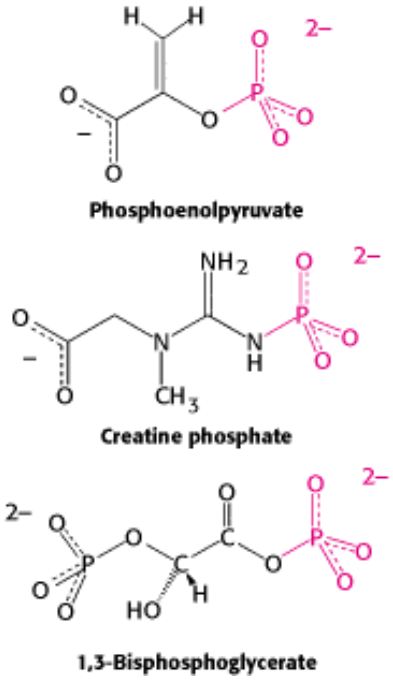


Figure 14.6. High Phosphoryl Transfer Potential Compounds. These compounds have a higher phosphoryl transfer potential than that of ATP and can be used to phosphorylate ADP to form ATP.

Table 14.1. Standard free energies of hydrolysis of some phosphorylated compounds

Compound	kcal mol^{-1}	kJ mol^{-1}
Phosphoenolpyruvate	-14.8	-61.9
1,3-Bisphosphoglycerate	-11.8	-49.4
Creatine phosphate	-10.3	-43.1
ATP (to ADP)	- 7.3	-30.5
Glucose 1-phosphate	- 5.0	-20.9
Pyrophosphate	- 4.6	-19.3
Glucose 6-phosphate	- 3.3	-13.8
Glycerol 3-phosphate	- 2.2	- 9.2

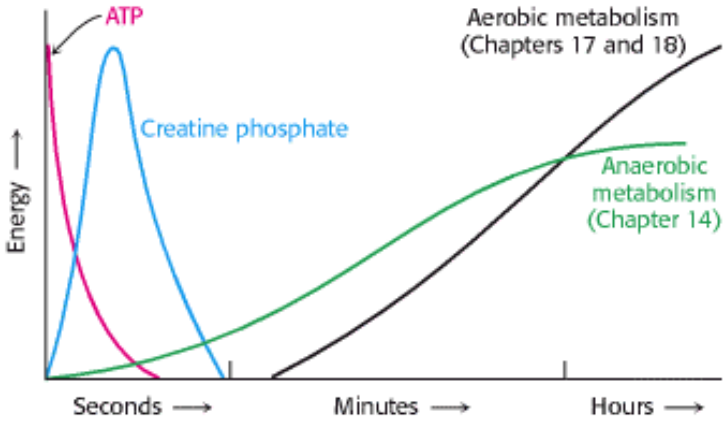


Figure 14.7. Sources of ATP During Exercise. In the initial seconds, exercise is powered by existing high phosphoryl transfer compounds (ATP and creatine phosphate). Subsequently, the ATP must be regenerated by metabolic pathways.

14.2. The Oxidation of Carbon Fuels Is an Important Source of Cellular Energy

ATP serves as the principal *immediate donor of free energy* in biological systems rather than as a long-term storage form of free energy. In a typical cell, an ATP molecule is consumed within a minute of its formation. Although the total quantity of ATP in the body is limited to approximately 100 g, *the turnover of this small quantity of ATP is very high*. For example, a resting human being consumes about 40 kg of ATP in 24 hours. During strenuous exertion, the rate of utilization of ATP may be as high as 0.5 kg/minute. For a 2-hour run, 60 kg (132 pounds) of ATP is utilized. Clearly, it is vital to have mechanisms for regenerating ATP. Motion, active transport, signal amplification, and biosynthesis can occur only if ATP is continually regenerated from ADP (Figure 14.8). The generation of ATP is one of the primary roles of catabolism. The carbon in fuel molecules—such as glucose and fats—is oxidized to CO_2, and the energy released is used to regenerate ATP from ADP and P_i.

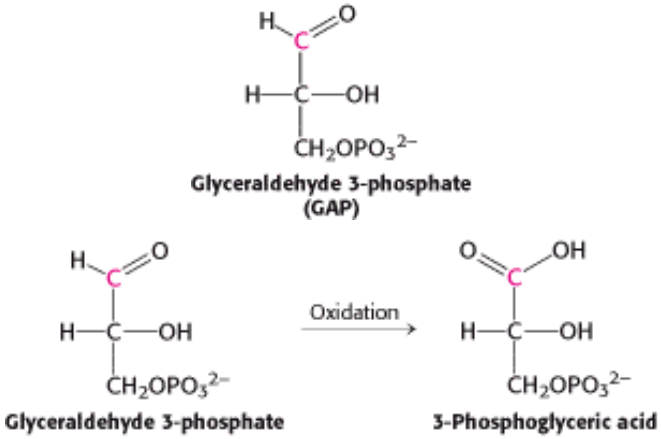
In aerobic organisms, the ultimate electron acceptor in the oxidation of carbon is O_2 and the oxidation product is CO_2. Consequently, the more reduced a carbon is to begin with, the more exergonic its oxidation will be. Figure 14.9 shows

the $\Delta G^{\circ\prime}$ of oxidation for one-carbon compounds.

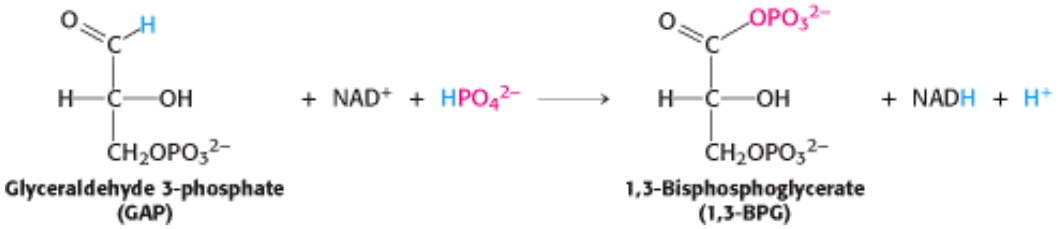
Although fuel molecules are more complex (Figure 14.10) than the singlecarbon compounds depicted in Figure 14.9, when a fuel is oxidized, the oxidation takes place one carbon at a time. The carbon oxidation energy is used in some cases to create a compound with high phosphoryl transfer potential and in other cases to create an ion gradient. In either case, the end point is the formation of ATP.

14.2.1. High Phosphoryl Transfer Potential Compounds Can Couple Carbon Oxidation to ATP Synthesis

How is the energy released in the oxidation of a carbon compound converted into ATP? As an example, consider glyceraldehyde 3-phosphate (shown in the margin), which is a metabolite of glucose formed in the oxidation of that sugar. The C-1 carbon (shown in red) is a component of an aldehyde and is not in its most oxidized state. Oxidation of the aldehyde to an acid will release energy.



However, the oxidation does not take place directly. Instead, the carbon oxidation generates an acyl phosphate, 1,3-bisphosphoglycerate. The electrons released are captured by NAD^+, which we will consider shortly.



For reasons similar to those discussed for ATP (Section 14.1.4), 1,3-bisphosphoglycerate has a high phosphoryl transfer potential. Thus, the cleavage of 1,3-BPG can be coupled to the synthesis of ATP.

The energy of oxidation is initially trapped as a high-energy phosphate compound and then used to form ATP. The oxidation energy of a carbon atom is transformed into phosphoryl transfer potential, first as 1,3-bisphosphoglycerate and ultimately as ATP. We will consider these reactions in mechanistic detail in Section 16.1.5.

14.2.2. Ion Gradients Across Membranes Provide an Important Form of Cellular Energy That Can Be Coupled to ATP Synthesis

The electrochemical potential of *ion gradients across membranes*, produced by the oxidation of fuel molecules or by photosynthesis, ultimately powers the synthesis of most of the ATP in cells. In general, ion gradients are versa-tile means of coupling thermodynamically unfavorable reactions to favorable ones. Indeed, in animals, *proton gradients* generated by the oxidation of carbon fuels account for more than 90% of ATP generation (Figure 14.11). This process is called *oxidative phosphorylation* (Chapter 18). ATP hydrolysis can then be used to form ion gradients of different types and functions. The electrochemical potential of a Na^+ gradient, for example, can be tapped to pump Ca^{2+} out of cells (Section 13.4) or to transport nutrients such as sugars and amino acids into cells.

14.2.3. Stages in the Extraction of Energy from Foodstuffs

Let us take an overall view of the processes of energy conversion in higher organisms before considering them in detail in subsequent chapters. Hans Krebs described three stages in the generation of energy from the oxidation of foodstuffs (Figure 14.12).

In the first stage, large molecules in food are broken down into smaller units. Proteins are hydrolyzed to their 20 kinds of constituent amino acids, polysaccharides are hydrolyzed to simple sugars such as glucose, and fats are hydrolyzed to glycerol and fatty acids. This stage is strictly a preparation stage; no useful energy is captured in this phase.

In the second stage, these numerous small molecules are degraded to a few simple units that play a central role in metabolism. In fact, most of them—sugars, fatty acids, glycerol, and several amino acids—are converted into the acetyl unit of acetyl CoA (Section 14.3.1). Some ATP is generated in this stage, but the amount is small compared with that obtained in the third stage.

In the third stage, ATP is produced from the complete oxidation of the acetyl unit of acetyl CoA. The third stage consists of the citric acid cycle and oxidative phosphorylation, which are *the final common pathways in the oxidation of fuel molecules*. Acetyl CoA brings acetyl units into the citric acid cycle [also called the tricarboxylic acid (TCA) cycle or Krebs cycle], where they are completely oxidized to CO_2. Four pairs of electrons are transferred (three to NAD^+ and one to FAD) for each acetyl group that is oxidized. Then, a proton gradient is generated as electrons flow from the reduced forms of these carriers to O_2, and this gradient is used to synthesize ATP.

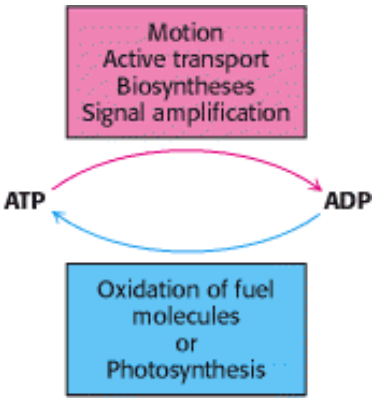


Figure 14.8. ATP-ADP Cycle. This cycle is the fundamental mode of energy exchange in biological systems.

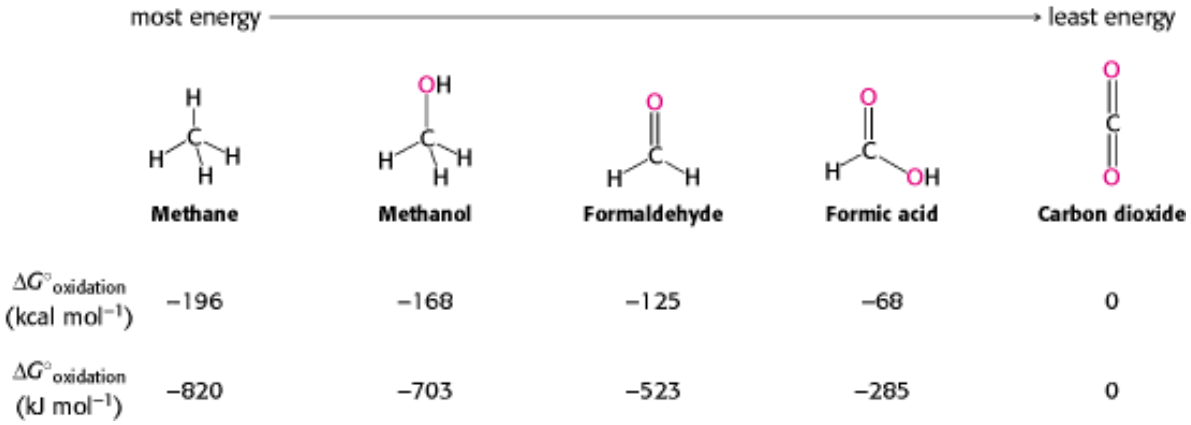


Figure 14.9. Free Energy of Oxidation of Single-Carbon Compounds.

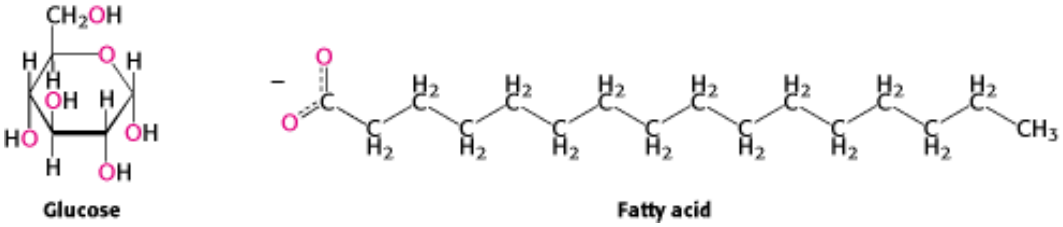


Figure 14.10. Prominent Fuels. Fats are a more efficient fuel source than carbohydrates such as glucose because the carbon in fats is more reduced.

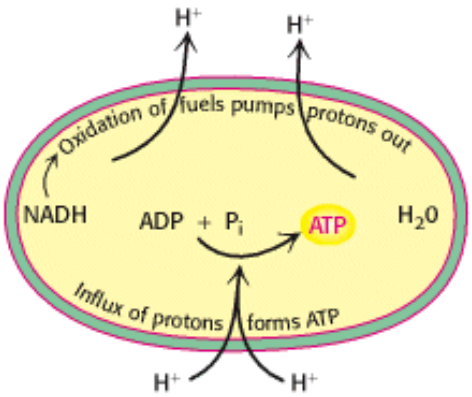


Figure 14.11. Proton Gradients. The oxidation of fuels can power the formation of proton gradients. These proton gradients can in turn drive the synthesis of ATP.

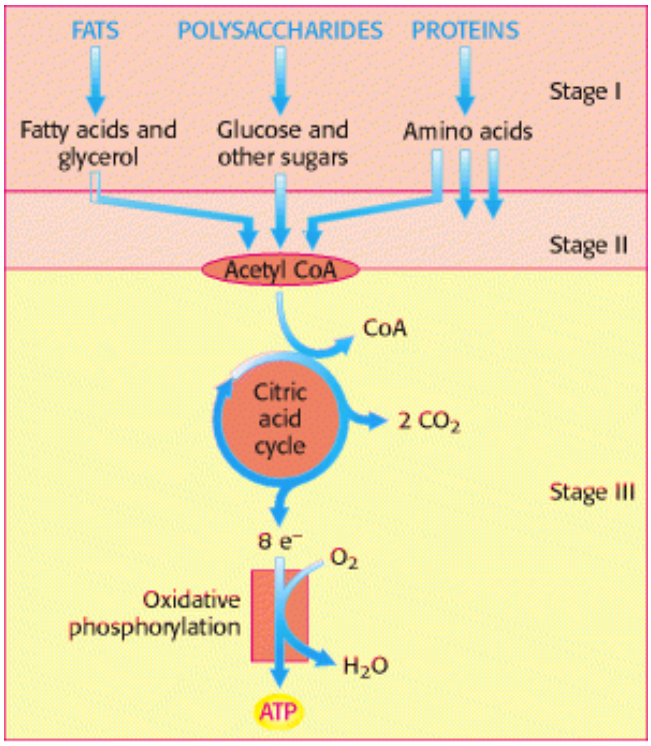


Figure 14.12. Stages of Catabolism. The extraction of energy from fuels can be divided into three stages.

14.3. Metabolic Pathways Contain Many Recurring Motifs

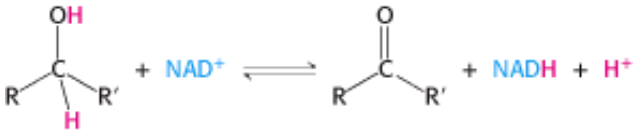
At first glance, metabolism appears intimidating because of the sheer number of reactants and reactions. Nevertheless, there are unifying themes that make the comprehension of this complexity more manageable. These unifying themes include common metabolites, reactions, and regulatory schemes that stem from a common evolutionary heritage.

14.3.1. Activated Carriers Exemplify the Modular Design and Economy of Metabolism

We have seen that phosphoryl transfer can be used to drive otherwise endergonic reactions, alter the energy of conformation of a protein, or serve as a signal to alter the activity of a protein. The phosphoryl-group donor in all of these reactions is ATP. In other words, *ATP is an activated carrier of phosphoryl groups because phosphoryl transfer from ATP is an exergonic process*. The use of activated carriers is a recurring motif in biochemistry, and we will consider several such carriers here.

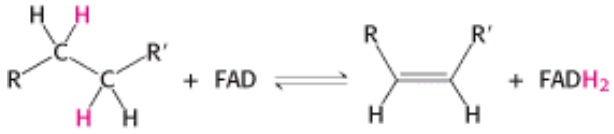
1. *Activated carriers of electrons for fuel oxidation*. In aerobic organisms, the ultimate electron acceptor in the oxidation of fuel molecules is O_2. However, electrons are not transferred directly to O_2. Instead, fuel molecules transfer electrons to special carriers, which are either *pyridine nucleotides* or *flavins*. The reduced forms of these carriers then transfer their highpotential electrons to O_2.

Nicotinamide adenine dinucleotide is a major electron carrier in the oxidation of fuel molecules (Figure 14.13). The reactive part of NAD^+ is its nicotinamide ring, a pyridine derivative synthesized from the vitamin niacin. *In the oxidation of a substrate, the nicotinamide ring of NAD $^+$ accepts a hydrogen ion and two electrons, which are equivalent to a hydride ion*. The reduced form of this carrier is called *NADH*. In the oxidized form, the nitrogen atom carries a positive charge, as indicated by NAD^+. NAD^+ is the electron acceptor in many reactions of the type



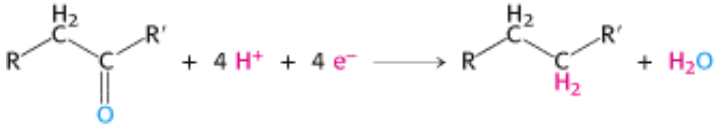
In this dehydrogenation, one hydrogen atom of the substrate is directly transferred to NAD^+, whereas the other appears in the solvent as a proton. Both electrons lost by the substrate are transferred to the nicotinamide ring.

The other major electron carrier in the oxidation of fuel molecules is the coenzyme *flavin adenine dinucleotide* (Figure 14.14). The abbreviations for the oxidized and reduced forms of this carrier are FAD and $FADH_2$, respectively. FAD is the electron acceptor in reactions of the type



The reactive part of FAD is its isoalloxazine ring, a derivative of the vitamin riboflavin (Figure 14.15). FAD, like NAD^+, can accept two electrons. In doing so, FAD, unlike NAD^+, takes up two protons. These carriers of high-potential electrons as well as flavin mononucleotide (FMN), an electron carrier related to FAD, will be considered further in Chapter 18.

2. *An activated carrier of electrons for reductive biosynthesis*. High-potential electrons are required in most biosyntheses because the precursors are more oxidized than the products. Hence, reducing power is needed in addition to ATP. For example, in the biosynthesis of fatty acids, the keto group of an added two-carbon unit is reduced to a methylene group in several steps. This sequence of reactions requires an input of four electrons.

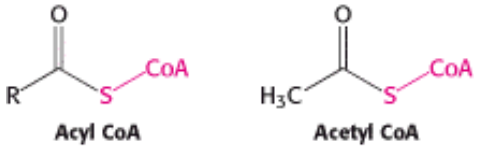


The electron donor in most reductive biosyntheses is NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$; see Figure 14.13). NADPH differs from NADH in that the 2′-hydroxyl group of its adenosine moiety is esterified with phosphate. NADPH carries electrons in the same way as NADH. However, *NADPH is used almost exclusively for reductive biosyntheses, whereas NADH is used primarily for the generation of ATP*. The extra phosphoryl group on NADPH is a tag that enables enzymes to distinguish between high-potential electrons to be used in anabolism and those to be used in catabolism.

3. *An activated carrier of two-carbon fragments*. Coenzyme A, another central molecule in metabolism, is a carrier of acyl groups (Figure 14.16). Acyl groups are important constituents both in catabolism, as in the oxidation of fatty acids, and in anabolism, as in the synthesis of membrane lipids. The terminal sulfhydryl group in CoA is the reactive site. Acyl groups are linked to CoA by thioester bonds. The resulting derivative is called an *acyl CoA*. An acyl group often linked to CoA is the acetyl unit; this derivative is called *acetyl CoA*. The $\Delta G^{\circ\prime}$ for the hydrolysis of acetyl CoA has a large

negative value:

$$\text{Acetyl CoA} + H_2O \rightleftharpoons \text{acetate} + \text{CoA} + H^+ \qquad \Delta G^{\circ\prime} = -7.5 \text{ kcal mol}^{-1} (-31.4 \text{ kJ mol}^{-1})$$



The hydrolysis of a thioester is thermodynamically more favorable than that of an oxygen ester because the electrons of the C=O bond cannot form resonance structures with the C—S bond that are as stable as those that they can form with the C—O bond. Consequently, *acetyl CoA has a high acetyl potential (acetyl group-transfer potential) because transfer of the acetyl group is exergonic.* Acetyl CoA carries an activated acetyl group, just as ATP carries an activated phosphoryl group.

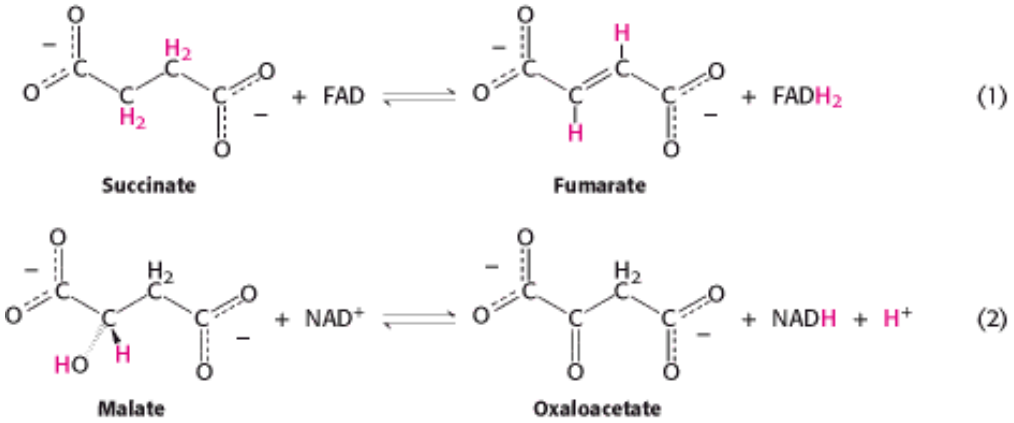
The use of activated carriers illustrates two key aspects of metabolism. First, NADH, NADPH, and $FADH_2$ react slowly with O_2 in the absence of a catalyst. Likewise, ATP and acetyl CoA are hydrolyzed slowly (in times of many hours or even days) in the absence of a catalyst. These molecules are kinetically quite stable in the face of a large thermodynamic driving force for reaction with O_2 (in regard to the electron carriers) and H_2O (in regard to ATP and acetyl CoA). *The kinetic stability of these molecules in the absence of specific catalysts is essential for their biological function because it enables enzymes to control the flow of free energy and reducing power*.

Second, *most interchanges of activated groups in metabolism are accomplished by a rather small set of carriers* (Table 14.2). The existence of a recurring set of activated carriers in all organisms is one of the unifying motifs of biochemistry. Furthermore, it illustrates the modular design of metabolism. A small set of molecules carries out a very wide range of tasks. Metabolism is readily comprehended because of the economy and elegance of its underlying design.

14.3.2. Key Reactions Are Reiterated Throughout Metabolism

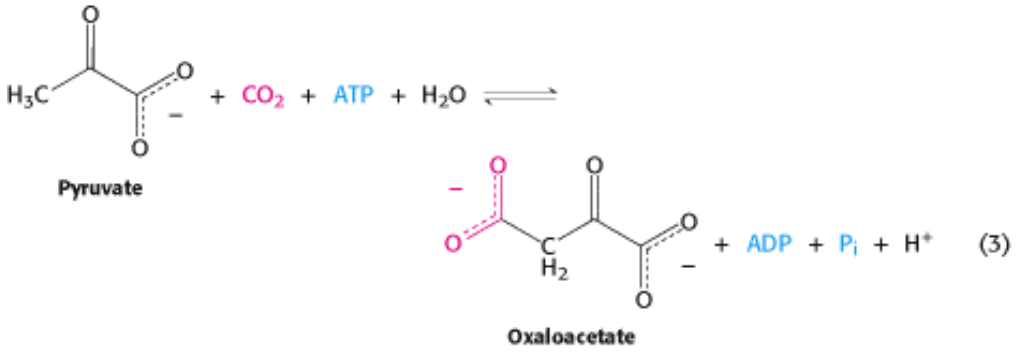
Just as there is an economy of design in the use of activated carriers, so is there an economy of design in biochemical reactions. The thousands of metabolic reactions, bewildering at first in their variety, can be subdivided into just six types (Table 14.3). Specific reactions of each type appear repeatedly, further reducing the number of reactions necessary for the student to learn.

1. *Oxidation-reduction reactions* are essential components of many pathways. Useful energy is often derived from the oxidation of carbon compounds. Consider the following two reactions:



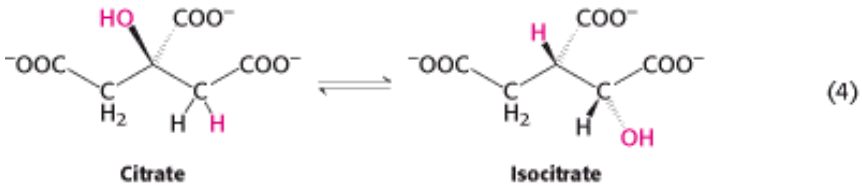
These two oxidation-reduction reactions are components of the citric acid cycle (Chapter 17), which completely oxidizes the activated two-carbon fragment of acetyl CoA to two molecules of CO_2. In reaction 1, $FADH_2$ carries the electrons, whereas, in reaction 2, electrons are carried by NADH. In biosynthetic oxidation-reduction reactions, NADPH is the reductant.

2. *Ligation reactions* form bonds by using free energy from ATP cleavage. Reaction 3 illustrates the ATP-dependent formation of a carbon-carbon bond, necessary to combine smaller molecules to form larger ones. Oxaloacetate is formed from pyruvate and CO_2.



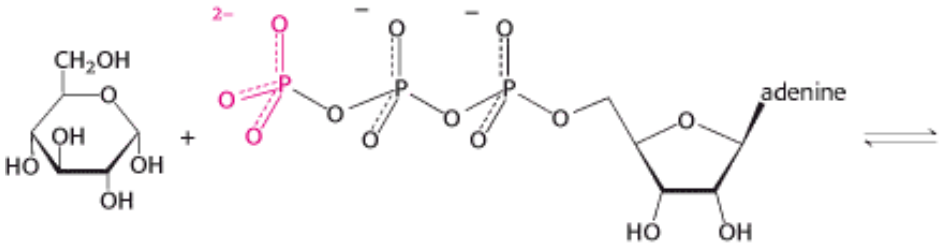
The oxaloacetate can be used in the citric acid cycle or converted into amino acids such as aspartic acid.

3. *Isomerization reactions* rearrange particular atoms within the molecule. Their role is often to prepare a molecule for subsequent reactions such as the oxidation-reduction reactions described in point 1.

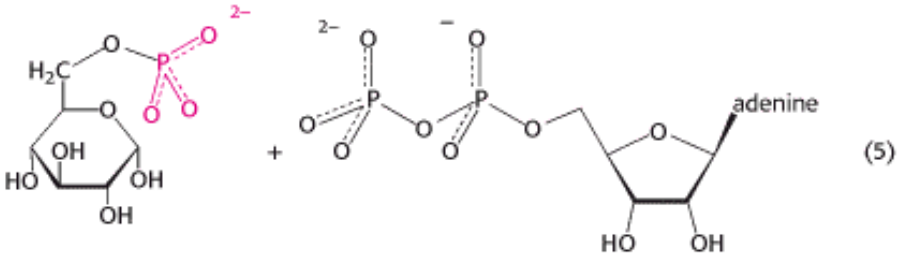


Reaction 4 is, again, a component of the citric acid cycle. This isomerization prepares the molecule for subsequent oxidation and decarboxylation by moving the hydroxyl group of citrate from a tertiary to a secondary position.

4. *Group-transfer reactions* play a variety of roles. Reaction 5 is representative of such a reaction. A phosphoryl group is transferred from the activated phosphoryl-group carrier, ATP, to glucose. This reaction traps glucose in the cell so that further catabolism can take place.



Glucose ATP

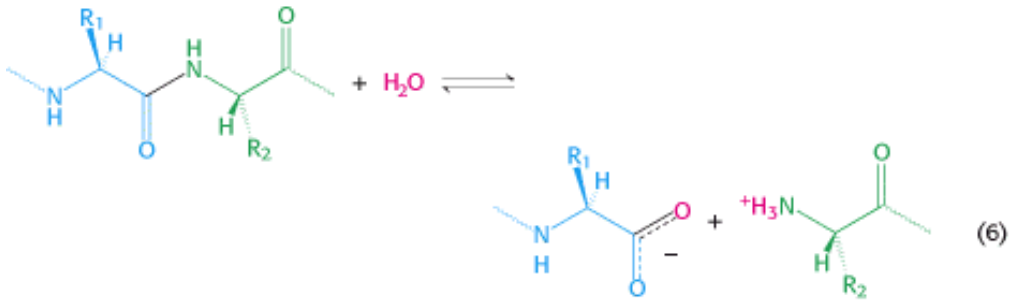


(5)

Glucose 6-phosphate (G-6P) ADP

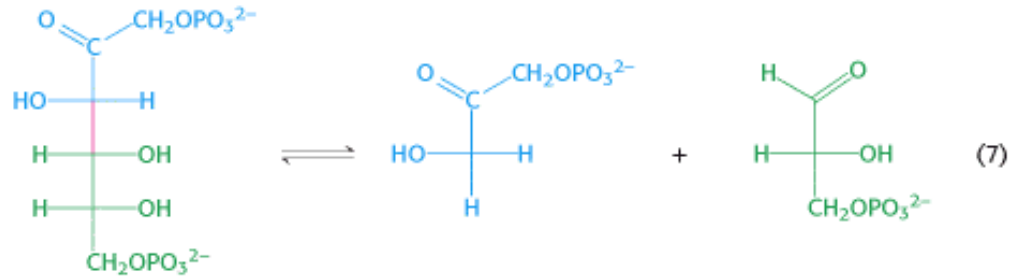
We saw earlier that group-transfer reactions are used to synthesize ATP (14.2.1). We will also see examples of their use in signaling pathways (Chapter 15).

5. *Hydrolytic reactions* cleave bonds by the addition of water. Hydrolysis is a common means employed to break down large molecules, either to facilitate further metabolism or to reutilize some of the components for biosynthetic purposes. Proteins are digested by hydrolytic cleavage (Chapters 9 and 10). Reaction 6 illustrates the hydrolysis of a peptide to yield two smaller peptides.



(6)

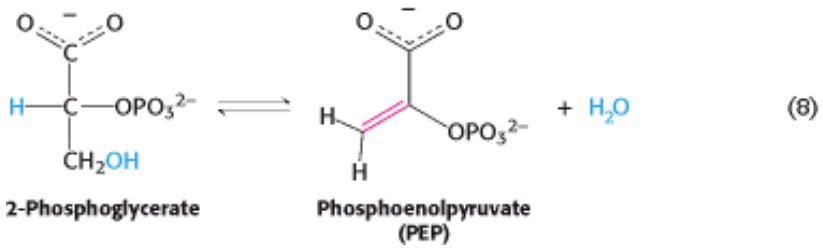
6. *The addition of functional groups to double bonds or the removal of groups to form double bonds* constitutes the final class of reactions. The enzymes that catalyze these types of reaction are classified as lyases (Section 8.1.3). An important example, illustrated in reaction 7, is the conversion of the six-carbon molecule fructose 1,6-bisphosphate (F-1, 6-BP) into 2 three-carbon fragments: dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.



(7)

Fructose 1,6-bisphosphate (F-1,6-BP) Dihydroxyacetone phosphate (DHAP) Glyceraldehyde 3-phosphate (GAP)

This reaction is a key step in glycolysis, a key pathway for extracting energy from glucose (Section 16.1.3). Dehydrations to form double bonds, such as the formation of phosphoenolpyruvate (Table 14.1) from 2-phosphoglycerate (reaction 8), are important reactions of this type.



The dehydration sets up the next step in the pathway, a group-transfer reaction that uses the that uses the high phosphoryl transfer potential of the product PEP to form ATP from ADP.

These six fundamental reaction types are the basis of metabolism. Remember that all six types can proceed in either direction, depending on the standard free energy for the specific reaction and the concentration of the reactants and products inside the cell. As an example of how simple themes are reiterated, consider the reactions shown in Figure 14.17. The same sequence of reactions is employed in the citric acid cycle, fatty acid degradation, the degradation of the amino acid lysine, and (in reverse) the biosynthesis of fatty acids. An effective way to learn is to look for commonalties in the diverse metabolic pathways that we will be studying. There is a chemical logic that, when exposed, renders the complexity of the chemistry of living systems more manageable and reveals its elegance.

14.3.3. Metabolic Processes Are Regulated in Three Principal Ways

It is evident that the complex network of reactions constituting intermediary metabolism must be rigorously regulated. At the same time, metabolic control must be flexible, because the external environments of cells are not constant. Metabolism is regulated by controlling (1) *the amounts of enzymes*, (2) *their catalytic activities*, and (3) *the accessibility of substrates*. The amount of a particular enzyme depends on both its rate of synthesis and its rate of degradation. The level of most enzymes is adjusted primarily by changing the *rate of transcription* of the genes encoding them. In *E. coli*, the presence of lactose, for example, induces within minutes a more than 50-fold increase in the rate of synthesis of β-galactosidase, an enzyme required for the breakdown of this disaccharide.

The catalytic activity of enzymes is controlled in several ways. *Reversible allosteric control* is especially important. For example, the first reaction in many biosynthetic pathways is allosterically inhibited by the ultimate product of the pathway. The inhibition of aspartate transcarbamoylase by cytidine triphosphate (Section 10.1) is a well-understood example of *feedback inhibition*. This type of control can be almost instantaneous. Another recurring mechanism is *reversible covalent modification*. For example, glycogen phosphorylase, the enzyme catalyzing the breakdown of glycogen, a storage form of sugar, is activated by phosphorylation of a particular serine residue when glucose is scarce (Section 21.2.1).

Hormones coordinate metabolic relations between different tissues, often by regulating the reversible modification of key enzymes. Hormones such as epinephrine trigger signal transduction cascades that lead to highly amplified changes in metabolic patterns in target tissues such as the muscle (Section 15.0.1). The hormone insulin promotes the entry of glucose into many kinds of cells. As will be discussed again in Chapter 15, many hormones act through intracellular messengers, such as cyclic AMP and calcium ion, that coordinate the activities of many target proteins.

Controlling the *flux of substrates* also regulates metabolism. The transfer of substrates from one compartment of a cell to another (e.g., from the cytosol to mitochondria) can serve as a control point.

An important general principle of metabolism is that *biosynthetic and degradative pathways are almost always distinct*.

This separation is necessary for energetic reasons, as will be evident in subsequent chapters. It also facilitates the control of metabolism. In eukaryotes, metabolic regulation and flexibility also are enhanced by compartmentalization. For example, fatty acid oxidation takes place in mitochondria, whereas fatty acid synthesis takes place in the cytosol. *Compartmentalization segregates opposed reactions.*

Many reactions in metabolism are controlled by the *energy status* of the cell. One index of the energy status is the *energy charge*, which is proportional to the mole fraction of ATP plus half the mole fraction of ADP, given that ATP contains two anhydride bonds, whereas ADP contains one. Hence, the energy charge is defined as

$$\text{Energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

The energy charge can have a value ranging from 0 (all AMP) to 1 (all ATP). Daniel Atkinson showed that *ATP-generating (catabolic) pathways are inhibited by an energy charge, whereas ATP-utilizing (anabolic) pathways are stimulated by a high-energy charge*. In plots of the reaction rates of such pathways versus the energy charge, the curves are steep near an energy charge of 0.9, where they usually intersect (Figure 14.18). It is evident that the control of these pathways has evolved to maintain the energy charge within rather narrow limits. In other words, *the energy charge, like the pH of a cell, is buffered*. The energy charge of most cells ranges from 0.80 to 0.95. An alternative index of the energy status is the *phosphorylation potential*, which is defined as

$$\text{Phosphorylation potential} = \frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]}$$

The phosphorylation potential, in contrast with the energy charge, depends on the concentration of P_i and is directly related to the free energy-storage available from ATP.

14.3.4. Evolution of Metabolic Pathways

How did the complex pathways that constitute metabolism evolve? This question, a difficult one to address, was approached in Chapter 2. The current thinking is that RNA was an early biomolecule and that, in an early RNA world, RNA served as catalysts and information-storage molecules (Section 2.2.2).

Why do activated carriers such as ATP, NADH, $FADH_2$, and coenzyme A contain adenosine diphosphate units (Figure 14.19)? A possible explanation is that these molecules evolved from the early RNA catalysts. Non-RNA units such as the isoalloxazine ring may have been recruited to serve as efficient carriers of activated electrons and chemical units, a function not readily performed by RNA itself. We can picture the adenine ring of $FADH_2$ binding to a uracil unit in a niche of an RNA enzyme (ribozyme) by base-pairing, whereas the isoalloxazine ring protrudes and functions as an electron carrier. When the more versatile proteins replaced RNA as the major catalysts, the ribonucleotide coenzymes stayed essentially unchanged because they were already well suited to their metabolic roles. The nicotin amide unit of NADH, for example, can readily transfer electrons irrespective of whether the adenine unit interacts with a base in an RNA enzyme or with amino acid residues in a protein enzyme. With the advent of protein enzymes, these important cofactors evolved as free molecules without losing the adenosine diphosphate vestige of their RNA-world ancestry. That molecules and motifs of metabolism are common to all forms of life testifies to their common origin and to the retention of functioning modules through billions of years of evolution. Our understanding of metabolism, like that of other biological processes, is enriched by inquiry into how these beautifully integrated patterns of reactions came into being.

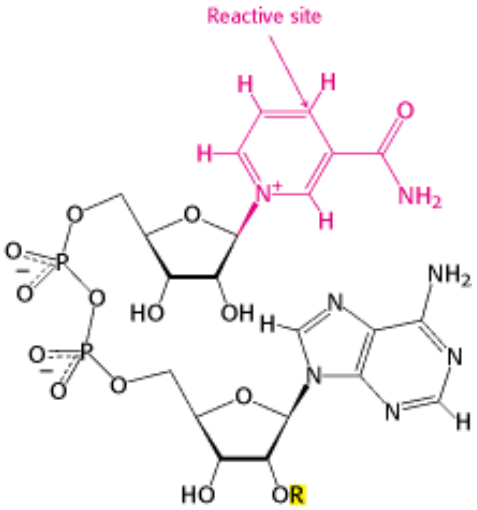


Figure 14.13. Structures of the Oxidized Forms of Nicotinamide-Derived Electron Carriers. Nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) are prominent carriers of high-energy electrons. In NAD^+, R = H; in $NADP^+$, R = PO_3^{2-}.

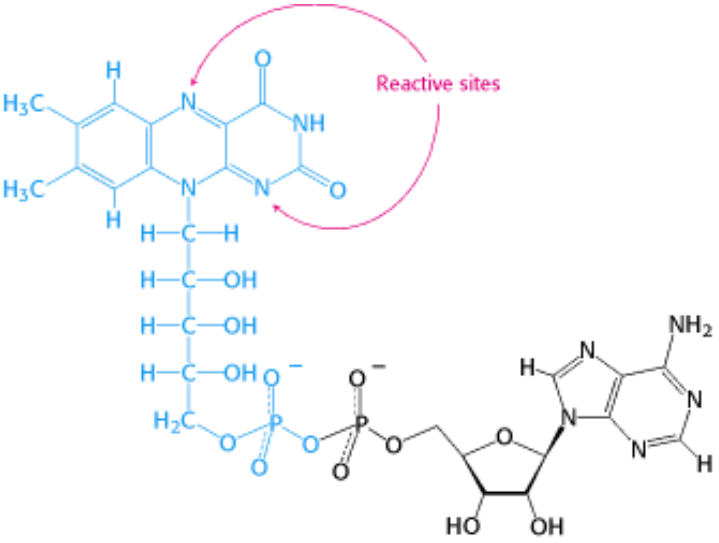
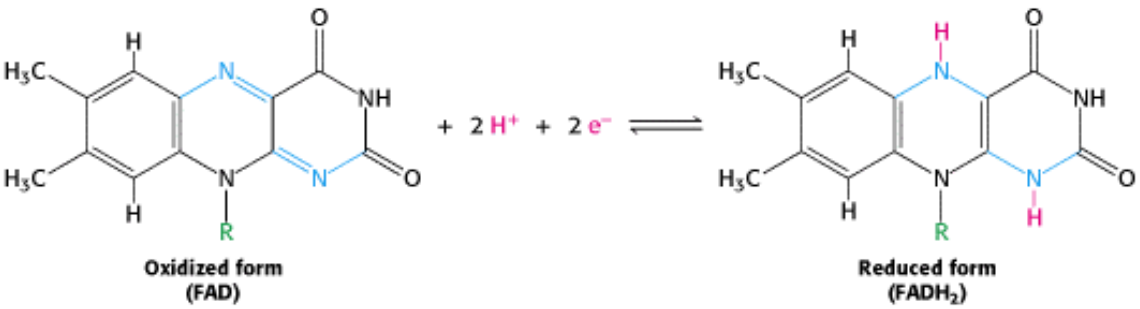


Figure 14.14. Structure of the Oxidized Form of Flavin Adenine Dinucleotide (FAD). This electron carrier consists of a flavin mononucleotide (FMN) unit (shown in blue) and an AMP unit (shown in black).



Oxidized form (FAD) Reduced form ($FADH_2$)

Figure 14.15. Structures of the Reactive Parts of FAD and $FADH_2$. The electrons and protons are carried by the isoalloxazine ring component of FAD and $FADH_2$.

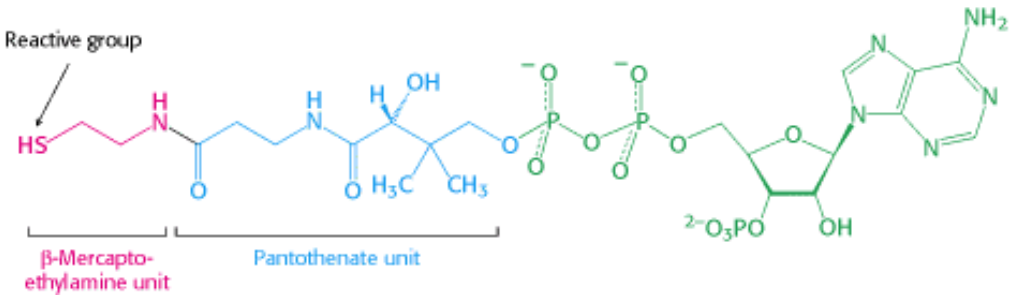


Figure 14.16. Structure of Coenzyme a (CoA).

Table 14.2. Some activated carriers in metabolism

Carrier molecule in activated form	Group carried	Vitamin precursor
ATP	Phosphoryl	
NADH and NADPH	Electrons	Nicotinate (niacin)
$FADH_2$	Electrons	Riboflavin (vitamin B_2)
$FMNH_2$	Electrons	Riboflavin (vitamin B_2)
Coenzyme A	Acyl	Pantothenate
Lipoamide	Acyl	
Thiamine pyrophosphate	Aldehyde	Thiamine (vitamin B_1)
Biotin	CO_2	Biotin
Tetrahydrofolate	One-carbon units	Folate
S-Adenosylmethionine	Methyl	
Uridine diphosphate glucose	Glucose	
Cytidine diphosphate diacylglycerol	Phosphatidate	
Nucleoside triphosphates	Nucleotides	

Note: Many of the activated carriers are coenzymes that are derived from water-soluble vitamins (Section 8.6.1).

Table 14.3. Types of chemical reactions in metabolism

Type of reaction	Description
Oxidation-reduction	Electron transfer
Ligation requiring ATP cleavage	Formation of covalent bonds (i.e., carbon-carbon bonds)
Isomerization	Rearrangement of atoms to form isomers
Group transfer	Transfer of a functional group from one molecule to another
Hydrolytic	Cleavage of bonds by the addition of water

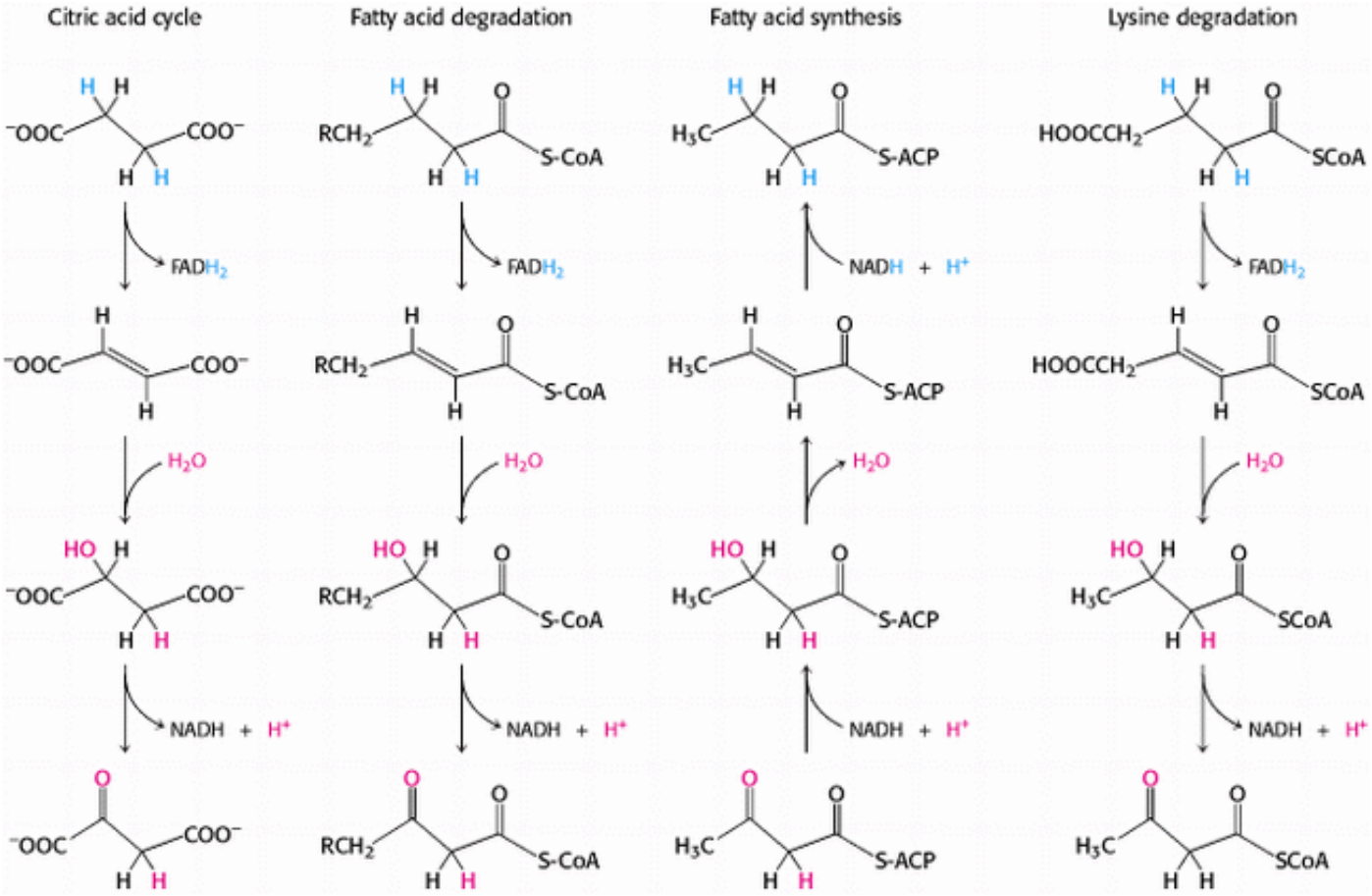


Figure 14.17. Metabolic Motifs. Some metabolic pathways have similar sequences of reactions in common—in this case, an oxidation, the addition of a functional group (from a water molecule) to a double bond, and another oxidation. ACP designates acyl carrier protein.

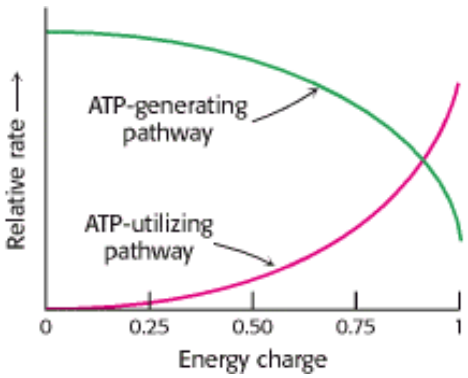


Figure 14.18. Energy Charge Regulates Metabolism. High concentrations of ATP inhibit the relative rates of a typical ATP-generating (catabolic) pathway and stimulate the typical ATP-utilizing (anabolic) pathway.

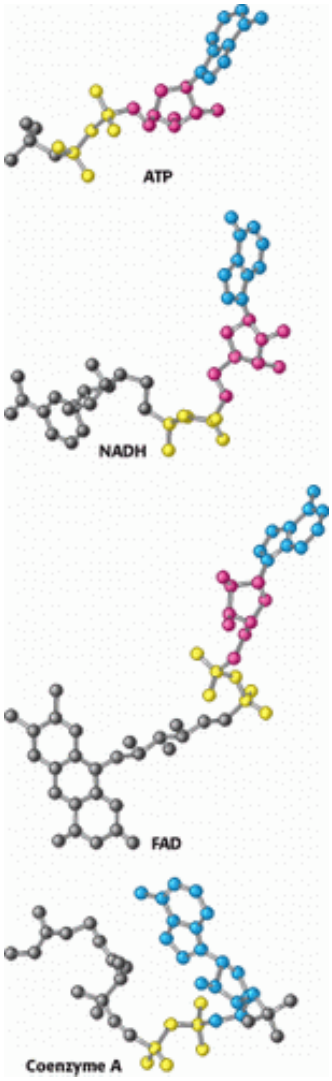


Figure 14.19. Adenosine Diphosphate (ADP) Is an Ancient Module in Metabolism. This fundamental building block is present in key molecules such as ATP, NADH, FAD, and coenzyme A. The adenine unit is shown in blue, the ribose unit in red, and the diphosphate unit in yellow.

Summary

All cells transform energy. They extract energy from their environment and use this energy to convert simple molecules into cellular components.

Metabolism Is Composed of Many Coupled, Interconnecting Reactions

The process of energy transduction takes place through a highly integrated network of chemical reactions called metabolism. Metabolism can be subdivided into catabolism (reactions employed to extract energy from fuels) and anabolism (reactions that use this energy for biosynthesis). The most valuable thermodynamic concept for understanding bioenergetics is free energy. A reaction can occur spontaneously only if the change in free energy (ΔG) is negative. A thermodynamically unfavorable reaction can be driven by a thermodynamically favorable one, which in many cases is the hydrolysis of ATP. The hydrolysis of ATP shifts the equilibrium of a coupled reaction by a factor of about 10^8. ATP, the universal currency of energy in biological systems, is an energy-rich molecule because it contains two phosphoanhydride bonds.

The Oxidation of Carbon Fuels Is an Important Source of Cellular Energy

ATP formation is coupled to the oxidation of carbon fuels, either directly or through the formation of ion gradients. Photosynthetic organisms can use light to generate such gradients. ATP is consumed in muscle contraction and other motions of cells, in active transport, in signal transduction processes, and in biosyntheses. There are three stages in the extraction of energy from foodstuffs by aerobic organisms. In the first stage, large molecules are broken down into smaller ones, such as amino acids, sugars, and fatty acids. In the second stage, these small molecules are degraded to a few simple units that have a pervasive role in metabolism. One of them is the acetyl unit of acetyl CoA, a carrier of activated acyl groups. The third stage of metabolism is the citric acid cycle and oxidative phosphorylation, in which ATP is generated as electrons flow to O_2, the ultimate electron acceptor, and fuels are completely oxidized to CO_2.

Metabolic Pathways Contain Many Recurring Motifs

Metabolism is characterized by common motifs. A small number of activated carriers, such as ATP, NADH, and acetyl CoA, are used in many metabolic pathways. NADPH, which carries two electrons at a high potential, provides reducing power in the biosynthesis of cell components from more-oxidized precursors. ATP and NADPH are continually generated and consumed. Most transfers of activated groups in metabolism are mediated by a recurring set of carriers. Moreover, key reaction types are used repeatedly in metabolic pathways.

Metabolism is regulated in a variety of ways. The amounts of some critical enzymes are controlled by regulation of the rate of protein synthesis and degradation. In addition, the catalytic activities of many enzymes are regulated by allosteric interactions (as in feedback inhibition) and by covalent modification. The movement of many substrates into cells and subcellular compartments also is controlled. Distinct pathways for biosynthesis and degradation contribute to metabolic regulation. The energy charge, which depends on the relative amounts of ATP, ADP, and AMP, plays a role in metabolic regulation. A high-energy charge inhibits ATP-generating (catabolic) pathways, whereas it stimulates ATP-utilizing (anabolic) pathways.

Key Terms

phototroph

chemotroph

metabolism or intermediary metabolism

catabolism

anabolism

amphibolic pathway

coupled reaction

phosphoryl transfer potential

oxidative phosphorylation

activated carrier

oxidation-reduction reaction

ligation reaction

isomerization reaction

group-transfer reaction

hydrolytic reaction

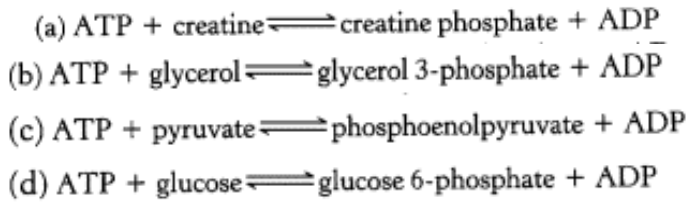
addition to or formation of double-bond reaction

energy charge

phosphorylation potential

Problems

1. *Energy flow*. What is the direction of each of the following reactions when the reactants are initially present in equimolar amounts? Use the data given in Table 14.1.



See answer

2. *A proper inference*. What information do the $\Delta G^{\circ\prime}$ data given in Table 14.1 provide about the relative rates of hydrolysis of pyrophosphate and acetyl phosphate?

See answer

3. *A potent donor*. Consider the following reaction:

(a) Calculate $\Delta G^{\circ\prime}$ and K'_{eq} at 25°C for this reaction, by using the data given in Table 14.1.

(b) What is the equilibrium ratio of pyruvate to phosphoenolpyruvate if the ratio of ATP to ADP is 10?

See answer

4. *Isomeric equilibrium*. Calculate $\Delta G^{\circ\prime}$ for the isomerization of glucose 6-phosphate to glucose 1-phosphate. What is the equilibrium ratio of glucose 6-phosphate to glucose 1-phosphate at 25°C?

See answer

5. *Activated acetate*. The formation of acetyl CoA from acetate is an ATP-driven reaction:

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{PP}_i$$

(a) Calculate $\Delta G^{\circ\prime}$ for this reaction by using data given in this chapter.

(b) The PP_i formed in the preceding reaction is rapidly hydrolyzed in vivo because of the ubiquity of inorganic pyrophosphatase. The $\Delta G^{\circ\prime}$ for the hydrolysis of PP_i is -4.6 kcal mol^{-1}. Calculate the $\Delta G^{\circ\prime}$ for the overall reaction. What effect does the hydrolysis of PP_i have on the formation of acetyl CoA?

See answer

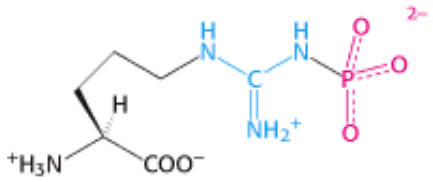
6. *Acid strength*. The p*K* of an acid is a measure of its proton-group-transfer potential.

(a) Derive a relation between $\Delta G^{\circ\prime}$ and p*K*.

(b) What is the $\Delta G^{\circ\prime}$ for the ionization of acetic acid, which has a p*K* of 4.8?

See answer

7. *Raison d'être*. The muscles of some invertebrates are rich in *arginine phosphate* (phosphoarginine). Propose a function for this amino acid derivative.



Arginine phosphate

See answer

8. *Recurring motif*. What is the structural feature common to ATP, FAD, NAD^+, and CoA?

See answer

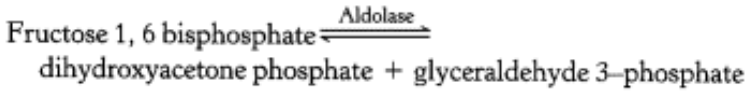
9. *Ergogenic help or hindrance?* Creatine is a popular, but untested, dietary supplement.

(a) What is the biochemical rationale for the use of creatine?

(b) What type of exercise would most benefit from creatine supplementation?

See answer

10. *Standard conditions versus real life*. The enzyme aldolase catalyzes the following reaction in the glycolytic pathway:



The $\Delta G^{\circ\prime}$ for the reaction is +5.7 kcal mol^{-1}, whereas the ΔG in the cell is -0.3 kcal mol^{-1}. Calculate the ratio of reactants to products under equilibrium and intracellular conditions. Using your results, explain how the reaction can be endergonic under standard conditions and exergonic under intracellular conditions.

See answer

11. *Not all alike*. The concentrations of ATP, ADP, and P_i differ with cell type. Consequently, the release of free energy with the hydrolysis of ATP will vary with cell type. Using the following table, calculate the ΔG for the hydrolysis of ATP in muscle, liver, and brain cells. In which cell type is the free energy of ATP hydrolysis greatest?

	ATP (mM)	ADP (mM)	P_i (mM)
Liver	3.5	1.8	5.0
Muscle	8.0	0.9	8.0
Brain	2.6	0.7	2.7

See answer

12. *Running downhill*. Glycolysis is a series of 10 linked reactions that convert one molecule of glucose into two molecules of pyruvate with the concomitant synthesis of two molecules of ATP (Chapter 16). The $\Delta G^{\circ\prime}$ for this set of reactions is -8.5 kcal mol^{-1} (-35.6 kJ mol^{-1}), whereas the ΔG is -18.3 kcal mol^{-1} (-76.6 kJ mol^{-1}). Explain why the free-energy release is so much greater under intracellular conditions than under standard conditions.

See answer

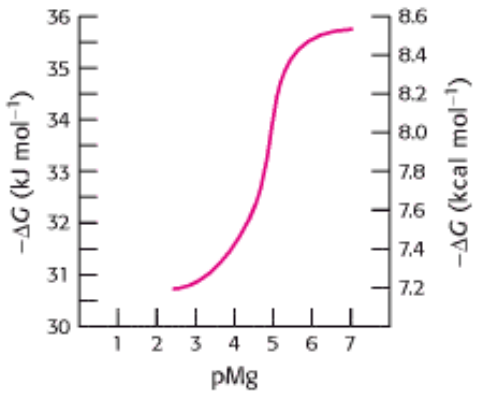
Chapter Integration Problem

13. *Activated sulfate*. Fibrinogen contains tyrosine-*O*-sulfate. Propose an activated form of sulfate that could react in vivo with the aromatic hydroxyl group of a tyrosine residue in a protein to form tyrosine-*O*-sulfate.

See answer

Data Interpretation

14. *Opposites attract*. The following graph shows how the ΔG for the hydrolysis of ATP varies as a function of the Mg^{2+} concentration (pMg = log $1/[Mg^{2+}]$).



(a) How does decreasing $[Mg^{2+}]$ affect the ΔG of hydrolysis for ATP?

(b) How can you explain this effect?

See answer

Media Problem

15. *Coupled reactions*. The most obvious role of enzymes is to accelerate reactions, but a second critical role is to couple reactions that would ordinarily be unrelated. From what you have learned from the text and the **Conceptual Insights** module on energetic coupling, explain why the coupling of chemical reactions in a single enzyme is critical for life.

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15. Signal-Transduction Pathways: An Introduction to Information Metabolism

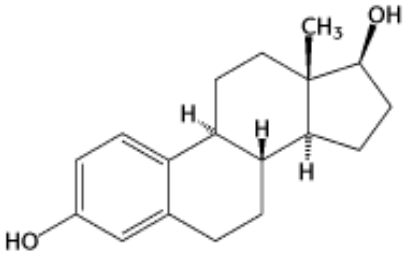
A cell is highly responsive to specific chemicals in its environment. Hormones are chemical signals that tell a cell to respond to a change in conditions. Molecules in food or aromas communicate taste and smell through their interaction with specialized sensory cells. This chapter provides an overview of *information metabolism* —how cells receive, process, and respond to information from the environment. The results of genome-sequencing efforts have underscored how widespread and diverse these information-processing circuits are. For example, approximately half of the 25 largest protein families encoded by the human genome deal primarily with information processing.

Signal-transduction cascades mediate the sensing and processing of stimuli. These molecular circuits detect, amplify, and integrate diverse external signals to generate responses such as changes in enzyme activity, gene expression, or ion-channel activity. This chapter is an introduction to some of the most important classes of molecules that participate in common signal-transduction pathways. We will encounter many specific pathways in their biochemical contexts in later chapters. In this chapter, we will also consider the consequences of defects in these pathways, particularly those leading to cancer.

15.0.1. Signal Transduction Depends on Molecular Circuits: An Overview

Signal-transduction pathways follow a broadly similar course that can be viewed as a molecular circuit (Figure 15.1). We begin by examining the challenges posed by transferring extracellular information to a cell's interior.

1. *Membrane receptors transfer information from the environment to the cell's interior.* A few nonpolar signal molecules such as estrogens and other steroid hormones are able to diffuse through the cell membranes and, hence, enter the cell. Once inside the cell, these molecules can bind to proteins that interact directly with DNA and modulate gene transcription. Thus, a chemical signal enters the cell and directly alters gene-expression patterns. These important signaling systems will be discussed in Chapter 31. However, most signal molecules are too large and too polar to pass through the membrane, and no appropriate transport systems are present. Thus, *the information that signal molecules are present must be transmitted across the cell membrane without the molecules themselves entering the cell.* A membrane-associated receptor protein often performs the function of information transfer across the membrane.



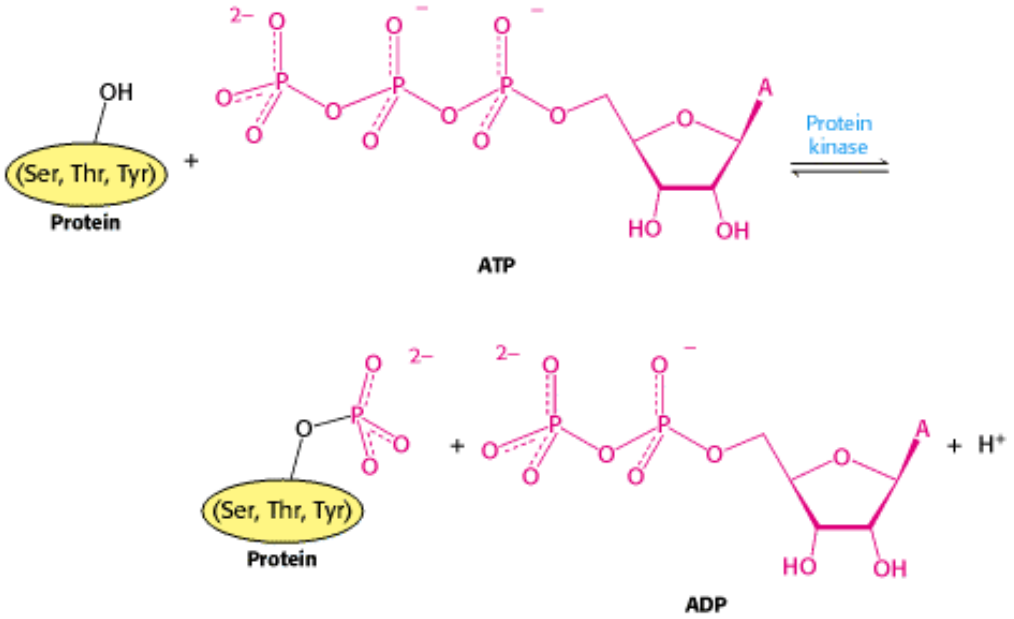
Estrogen
(estradiol)

Such a receptor is an intrinsic membrane protein that has both extracellular and intracellular domains. A binding site on the extracellular domain specifically recognizes the signal molecule (often referred to as the *ligand*). Such binding sites are analogous to enzyme active sites except that no catalysis takes place within them. The interaction of the ligand and the receptor alters the tertiary or quaternary structure of the receptor, including the intracellular domain. These structural changes are not sufficient to yield an appropriate response, because they are restricted to a small number of receptor molecules in the cell membrane. The information embodied by the presence of the ligand, often called the *primary messenger*, must be transduced into other forms that can alter the biochemistry of the cell.

2. *Second messengers relay information from the receptor-ligand complex.* Changes in the concentration of small molecules, called *second messengers*, constitute the next step in the molecular information circuit. Particularly important second messengers include cyclic AMP and cyclic GMP, calcium ion, inositol 1,4,5-trisphosphate, (IP_3), and diacylglycerol (DAG; Figure 15.2).

The use of second messengers has several consequences. First, second messengers are often free to diffuse to other compartments of the cell, such as the nucleus, where they can influence gene expression and other processes. Second, the signal may be amplified significantly in the generation of second messengers. Enzymes or membrane channels are almost always activated in second-messenger generation; each activated macromolecule can lead to the generation of many second messengers within the cell. Thus, *a low concentration of signal in the environment, even as little as a single molecule, can yield a large intracellular signal and response.* Third, the use of common second messengers in multiple signaling pathways creates both opportunities and potential problems. Input from several signaling pathways, often called *cross talk*, may affect the concentrations of common second messengers. Cross talk permits more finely tuned regulation of cell activity than would the action of individual independent pathways. However, inappropriate cross talk can cause second messengers to be misinterpreted.

3. *Protein phosphorylation is a common means of information transfer.* Many second messengers elicit responses by activating *protein kinases.* These enzymes transfer phosphoryl groups from ATP to specific serine, threonine, and tyrosine residues in proteins.



We previously encountered the cAMP-dependent protein kinase in Section 10.4.2. This protein kinase and others are the link that transduces changes in the concentrations of free second messengers into changes in the covalent structures of proteins. Although these changes are less transient than the changes in secondary-messenger concentrations, protein phosphorylation is not irreversible. Indeed, *protein phosphatases* are enzymes that hydrolytically remove specific phosphoryl groups from modified proteins.

4. *The signal is terminated.* Protein phosphatases are one mechanism for the termination of a signaling process. After a signaling process has been initiated and the information has been transduced to affect other cellular processes, the signaling processes must be terminated. Without such termination, cells lose their responsiveness to new signals. Moreover, signaling processes that fail to be terminated properly may lead to uncontrolled cell growth and the possibility of cancer.

Essentially every biochemical process presented in the remainder of this book either is a component of a signal-transduction pathway or can be affected by one. As we shall see, the use of *protein modules* in various combinations is a clear, even dominant, theme in the construction of signal-transduction proteins. Signal-transduction proteins have evolved by the addition of such ancillary modules to core domains to facilitate interactions with other proteins or cell membranes. By controlling which proteins interact with one another, these modules play important roles in determining the wiring diagrams for signal-transduction circuits.

We begin by considering the largest and one of the most important classes of receptor, the seven-transmembrane-helix receptors.

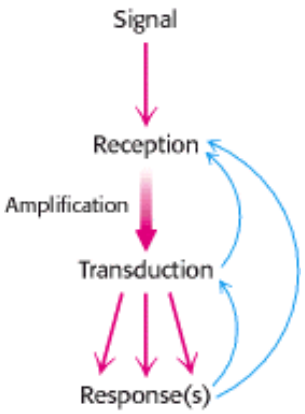


Figure 15.1. Principles of Signal Transduction. An environmental signal, such as a hormone, is first received by interaction with a cellular component, most often a cell-surface receptor. The information that the signal has arrived is then converted into other chemical forms, or *transduced*. The signal is often amplified before evoking a response. Feedback pathways regulate the entire signaling process.

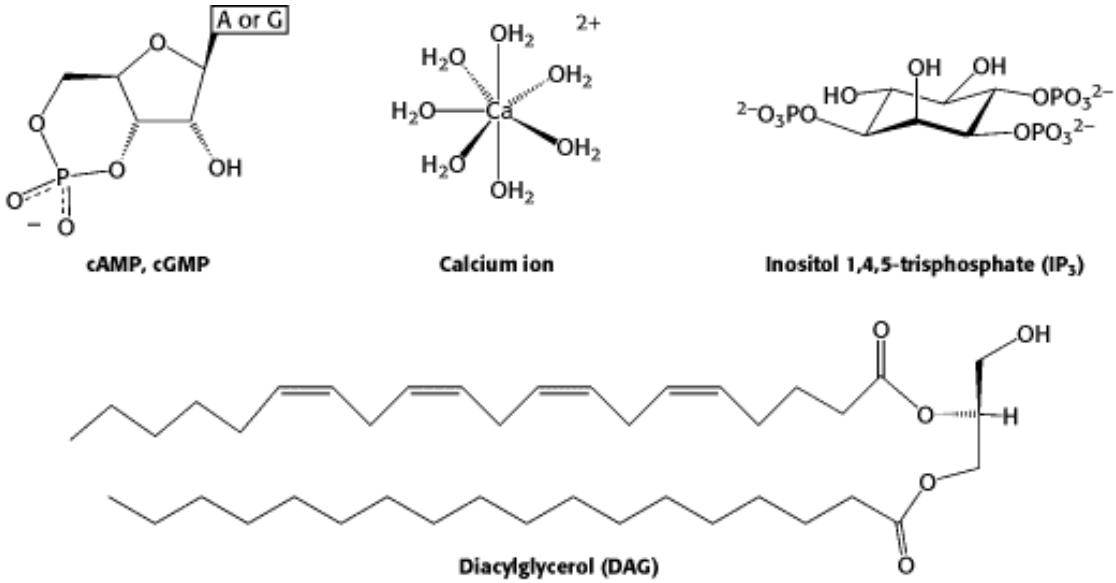
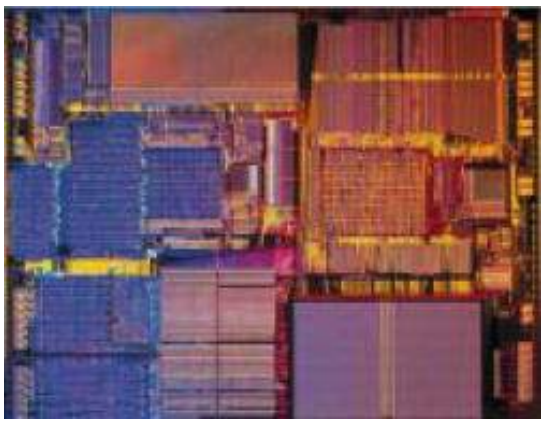
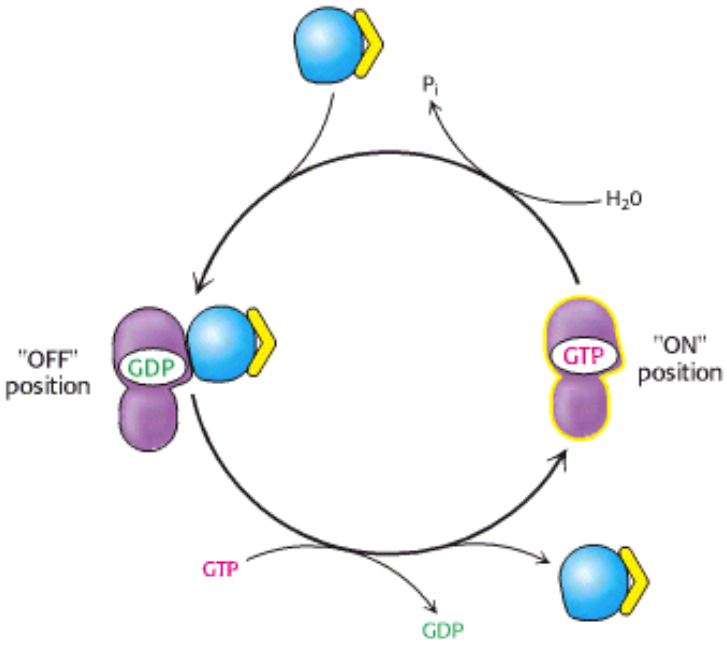


Figure 15.2. Common Second Messengers. Second messengers are intracellular molecules that change in concentration in response to environmental signals. That change in concentration conveys information inside the cell.



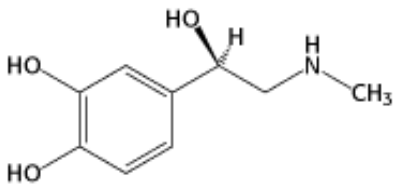


Molecular switches. Signal transduction circuits in biological systems have molecular on/off switches that, like those in a computer chip (above), transmit information when "on." Common among these are G proteins (right), which transmit a signal when bound to GTP and are silent when bound to GDP. [(Left) Courtesy of Intel.]

15.1. Seven-Transmembrane-Helix Receptors Change Conformation in Response to Ligand Binding and Activate G Proteins

Conceptual Insights, Signaling Pathways: Response and Recovery. Animations in this media module show how 7TM receptors and G proteins are employed in two sensory-system signaling pathways.

The *seven-transmembrane-helix (7TM) receptors* are responsible for transmitting information initiated by signals as diverse as photons, odorants, tastants, hormones, and neurotransmitters (Table 15.1). Several thousand such receptors are known, and the list continues to grow. As the name indicates, these receptors contain seven helices that span the membrane bilayer. The receptors are sometimes referred to as serpentine receptors because the single polypeptide chain "snakes" through the membrane seven times (Figure 15.3A). A well-characterized member of this family is *rhodopsin.* The "ligand" for this protein, which plays an essential role in vision, is a photon (Section 32.3.1). An example of a receptor that responds to chemical signals is the β-*adrenergic receptor*. This protein binds epinephrine (also called *adrenaline*), a hormone responsible for the "fight or flight" response. We will address the biochemical roles of this hormone in more detail later (Section 21.3.1).



Epinephrine

Recently, the three-dimensional structure of bovine rhodopsin was determined in its unactivated form (Figure 15.3B). A variety of evidence reveals that the 7TM receptors, particularly their cytoplasmic loops and their carboxyl termini, change conformation in response to ligand binding, although the details of these conformational changes remain to be established. Thus, *the binding of a ligand from outside the cell induces a conformational change in the 7TM receptor that can be detected inside the cell.* Even though vision and response to hormones would seem to have little in common,

a comparison of the amino acid sequences of rhodopsin and the β-adrenergic receptor clearly reveals homology. On the basis of this sequence comparison, the β-adrenergic receptor is expected to have a structure quite similar to that of rhodopsin. As we shall see, these receptors also have in common the next step in their signaling transduction cascades.

15.1.1. Ligand Binding to 7TM Receptors Leads to the Activation of G Proteins

What is the next step in the pathway after the binding of epinephrine by the β-adrenergic receptor? An important clue was Martin Rodbell's finding that GTP in addition to hormone is essential for signal transduction to proceed. Equally revealing was the observation that hormone binding stimulates GTP hydrolysis. These findings led to the discovery by Alfred Gilman that *a guanyl nucleotide-binding protein is an intermediary in signal transduction from the 7TM receptors.* This signal-coupling protein is termed a *G protein* (*G* for guanyl nucleotide). The activated G protein stimulates the activity of *adenylate cyclase,* an enzyme that increases the concentration of cAMP by forming it from ATP (Figure 15.4).

15.1.2. G Proteins Cycle Between GDP- and GTP-Bound Forms

How do these G proteins operate? *In the unactivated state, the guanyl nucleotide bound to the G protein is GDP.* In this form, the G protein exists as a *heterotrimer* consisting of α, β, and γ subunits; the α subunit (referred to as G_{α}) binds the nucleotide (Figure 15.5). The α subunit is a member of the P-loop NTPases family (Section 9.4.1) and the P-loop that participates in nucleotide binding. The β subunit contains a seven-bladed propeller structure, and the γ subunit comprises a pair of α helices that wrap around the β subunit (Figure 15.6). The α and γ subunits are usually anchored to the membrane by covalently attached fatty acids. *The role of the hormone-bound receptor is to catalyze the exchange of GTP for bound GDP.* The hormone-receptor complex interacts with the heterotrimeric G protein and opens the nucleotide-binding site so that GDP can depart and GTP from solution can bind. The α subunit simultaneously dissociates from the β γ dimer ($G_{\beta\gamma}$). The structure of the G_{α} subunit conforms tightly to the GTP molecule; in particular, three stretches of polypeptide (termed switch I, switch II, and switch III) interact either directly or indirectly with the γ phosphate of GTP (Figure 15.7). These structural changes are responsible for the reduced affinity of G_{α} for $G_{\beta\gamma}$. *The dissociation of the G-protein heterotrimer into G_{α} and $G_{\beta\gamma}$ units transmits the signal that the receptor has bound its ligand.* Moreover, the surfaces of G_{α} and $G_{\beta\gamma}$ that had formed the trimer interface are now exposed to interact with other proteins.

A single hormone-receptor complex can stimulate nucleotide exchange in many G-protein heterotrimers. Thus, hundreds of G_{α} molecules are converted from their GDP into their GTP forms for each bound molecule of hormone, giving an *amplified response.* All 7TM receptors appear to be coupled to G proteins, and so the 7TM receptors are sometimes referred to as *G-protein-coupled receptors* or *GPCRs.* Do all of the signals that function by means of 7TM receptors funnel through the same G protein? Indeed not. Different G proteins exist, and they can affect downstream targets in different ways when activated. For example, in regard to the G protein coupled to the β-adrenergic receptor, the α subunit binds to adenylate cyclase and stimulates its enzymatic activity. This subunit is referred to as G_{α^s}, the *s* in the subscript indicating the subunit's stimulatory role. The human genome contains more than 15 genes encoding the α subunits, 5 encoding the β subunits, and 10 encoding the γ subunits. Thus, in principle, there could be more than a thousand heterotrimeric G proteins; however, the number of combinations that actually exists is not known. Selected members of this family are shown in Table 15.2. Only a small subset of these proteins is expressed in a particular cell.

15.1.3. Activated G Proteins Transmit Signals by Binding to Other Proteins

The adenylate cyclase enzyme that is activated by the epinephrine-β-adrenergic receptor complex is a membrane protein that contains 12 presumed membrane-spanning helices. The enzymatically active part of the protein is formed from two large intracellular domains: one is located between transmembrane helices 6 and 7 and the other after the last

transmembrane helix. The structure was determined for a complex formed between $G_{\alpha s}$ bound to a GTP analog and protein fragments corresponding to the active adenylate cyclase (Figure 15.8). As expected, the G protein binds to adenylate cyclase through the surface that had bound the $\beta\gamma$ dimer when the G protein was in its GDP form. The activation of the G protein exposes this surface and subtly changes it so that it now binds the surface of adenylate cyclase in preference to $G_{\beta\gamma}$. The interaction of $G_{\alpha s}$ with adenylate cyclase favors a more catalytically active conformation of the enzyme, thus stimulating cAMP production. *The net result is that the binding of epinephrine to the receptor on the cell surface increases the rate of cAMP production inside the cell.*

15.1.4. G Proteins Spontaneously Reset Themselves Through GTP Hydrolysis

The ability to shut down signal-transduction pathways is as critical as the ability to turn them on. How is the signal initiated by activated 7TM receptors switched off? *G_{α} subunits have intrinsic GTPase activity,* hydrolyzing bound GTP to GDP and P_i. This hydrolysis reaction is slow, however, requiring from seconds to minutes and thus allowing the GTP form of G_{α} to activate downstream components of the signal-transduction pathway before GTP hydrolysis deactivates the subunit. In essence, *the bound GTP acts as a built-in clock that spontaneously resets the G_{α} subunit after a short time period.* After GTP hydrolysis and the release of P_i, the GDP-bound form of G_{α} then reassociates with $G_{\beta\gamma}$ to reform the heterotrimeric protein (Figure 15.9).

The hormone-bound activated receptor must be reset as well to prevent the continuous activation of G proteins. This resetting is accomplished by two processes (Figure 15.10). First, the hormone dissociates, returning the receptor to its initial, unactivated state. The likelihood that the receptor remains in its unbound state depends on the concentration of hormone. Second, the hormone-receptor complex is deactivated by the phosphorylation of serine and threonine residues in the carboxyl-terminal tail. In the example under consideration, β-*adrenergic receptor kinase* phosphorylates the carboxyl-terminal tail of the hormone-receptor complex but not the unoccupied receptor. Finally, the binding of β-*arrestin,* binds to the phosphorylated receptor and further diminishes its G-protein-activating ability. Phosphorylation and the binding of β-arrestin account for the *desensitization (adaptation)* of the receptor subsequent to prolonged exposure to epinephrine. The epinephrine-initiated cascade, like many other signal-transduction processes, has evolved to *respond to changes in the strength of stimuli rather than to their absolute level.* Adaptation is advantageous because it enables receptors to respond to changes in the level of stimuli over a wide range of background levels.

15.1.5. Cyclic AMP Stimulates the Phosphorylation of Many Target Proteins by Activating Protein Kinase A

Let us continue to follow the information flow down this signal-transduction pathway. The increased concentration of cAMP can affect a wide range of cellular processes. For example, it enhances the degradation of storage fuels, increases the secretion of acid by the gastric mucosa, leads to the dispersion of melanin pigment granules, diminishes the aggregation of blood platelets, and induces the opening of chloride channels. How does cAMP influence so many cellular processes? Is there a common denominator for its diverse effects? Indeed there is. *Most effects of cyclic AMP in eukaryotic cells are mediated by activation of a single protein kinase.* This key enzyme is called *protein kinase A (PKA).* As discussed in Section 10.4.2, PKA consists of two regulatory (R) chains and two catalytic (C) chains. In the absence of cAMP, the R_2C_2 complex is catalytically inactive. The binding of cAMP to the regulatory chains releases the catalytic chains, which are enzymatically active on their own. Activated PKA then phosphorylates specific serine and threonine residues in many targets to alter their activity. The significance and far reach of the *adenylate cyclase cascade* are seen in the following examples:

1. In *glycogen metabolism* (Section 21.5), PKA phosphorylates two enzymes that lead to the breakdown of this polymeric store of glucose and the inhibition of further glycogen synthesis.

2. PKA stimulates the *expression of specific genes* by phosphorylating a transcriptional activator called the cAMP-

response element binding (CREB) protein (Section 31.3.6). This activity of PKA illustrates that signal-transduction pathways can extend into the nucleus to alter gene expression.

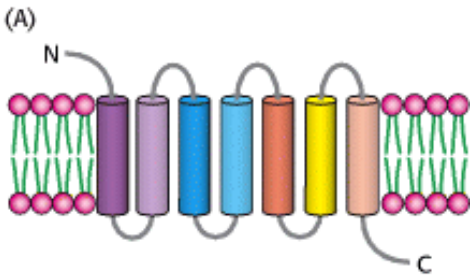
3. *Synaptic transmission* between pairs of neurons in *Aplysia* (a marine snail) is enhanced by serotonin, a neurotransmitter that is released by adjacent interneurons. Serotonin binds to a 7TM receptor to trigger an adenylate cyclase cascade. The rise in cAMP level activates PKA, which facilitates the closing of potassium channels by phosphorylating them. Closure of potassium channels increases the excitability of the target cell.

Thus, signal-transduction pathways that include 7TM receptors, the activation of adenylate cyclase, and the activation of PKA can modulate enzyme activities, gene-expression patterns, and membrane excitability.

Table 15.1. Biological functions mediated by 7TM receptors

- Smell
- Taste
- Vision
- Neurotransmission
- Hormone secretion
- Chemotaxis
- Exocytosis
- Control of blood pressure
- Embryogenesis
- Cell growth and differentiation
- Development
- Viral infection
- Carcinogenesis

Source: After J. S. Gutkind, *J. Biol. Chem.* 273(1998):1839.



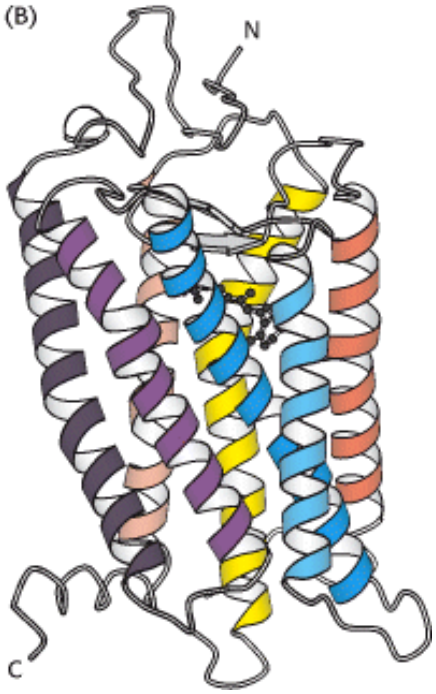


Figure 15.3. A 7TM Receptor. (A) Schematic representation of a 7TM receptor showing how it passes through the membrane seven times. (B) Three-dimensional structure of rhodopsin, a 7TM receptor taking part in visual signal transduction. As the first 7TM receptor whose structure has been determined, its structure provides a framework for understanding other 7TM receptors. A linked photoreceptor molecule, retinal, is present in the position where, in at least other 7TM receptors, ligands likely bind.

Figure 15.4. The β -Adrenergic Receptor Signal-Transduction Pathway. On binding of ligand, the receptor activates a G protein that in turn activates the enzyme adenylate cyclase. Adenylate cyclase generates the second messenger cAMP. The increase in cAMP results in a biochemical response to the initial signal.

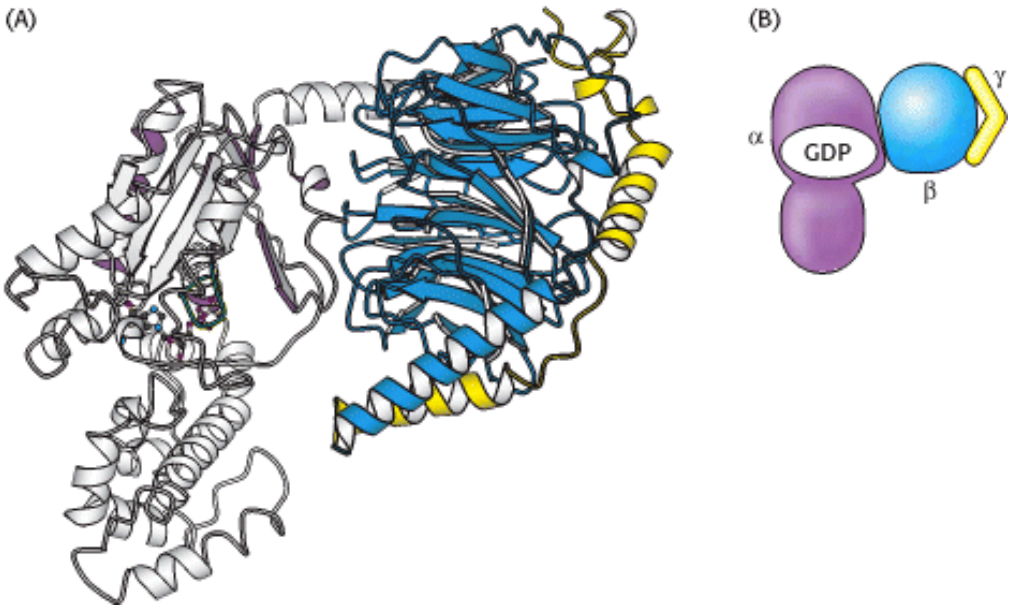


Figure 15.5. A Heterotrimeric G Protein. (A) A ribbon diagram shows the relation between the three subunits. In this complex, the α subunit (gray and purple) is bound to GDP. (B) A schematic representation of the heterotrimeric G protein.

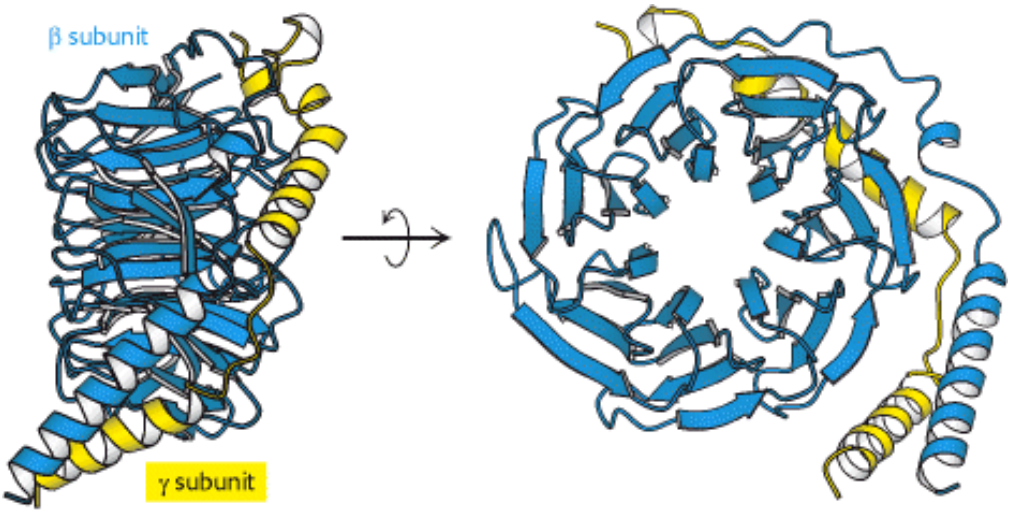


Figure 15.6. The β γ Subunits of the Heterotrimeric G Protein. Two views illustrate the interaction between the β and the γ subunits. The helices of the γ subunit (yellow) wrap around the β subunit (blue). The seven-bladed propeller structure of the β subunit is readily apparent in the representation on the right.

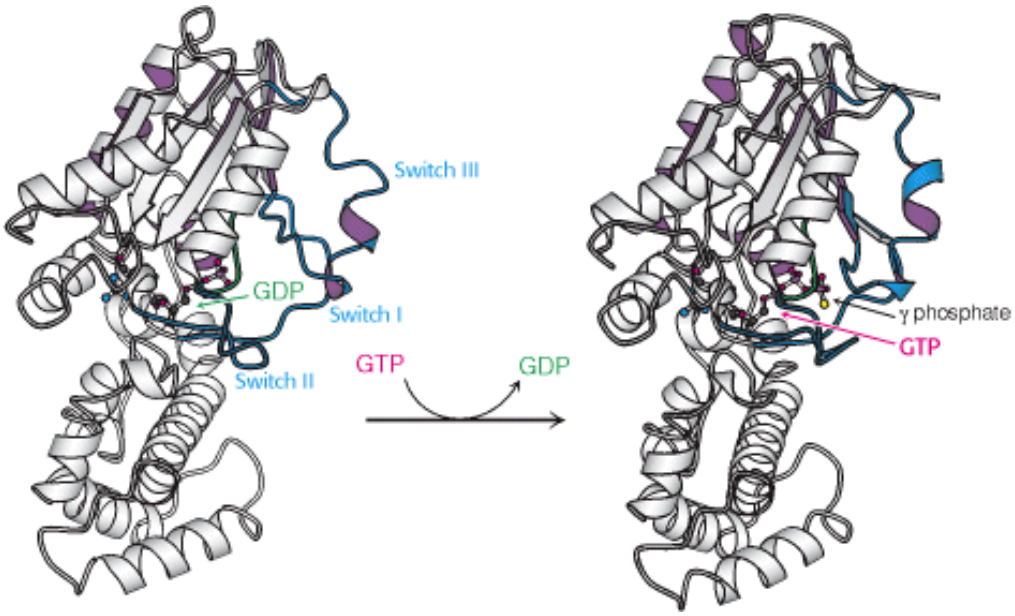


Figure 15.7. Conformational Changes in G_{α} On Nucleotide Exchange. (Left) Prior to activation, Gα binds GDP. (Right) On GTP for GDP exchanges, the three switch regions (shown in blue) close upon the nucleoside triphosphate, generating the active conformation.

Table 15.2. G-protein families and their functions

G_{α} class	Initiating signal	Downstream signal
G_{α^s}	β-Adrenergic amines, glucagon, parathyroid hormone, many others	Stimulates adenylate cyclase
G_{α^i}	Acetylcholine, α-adrenergic amines, many neurotransmitters	Inhibits adenylate cyclase
G_{α^t}	Photons	Stimulates cGMP phosphodiesterase
G_{α^q}	Acetylcholine, α-adrenergic amines, many neurotransmitters	Increases IP_3 and intracellular calcium
$G_{\alpha^{13}}$	Thrombin, other agonists	Stimulates Na^+ and H^+ exchange

Source: Z. Farfel, H. R. Bourne, and T. Iiri. *N. Engl. J. Med.* 340(1999):1012.

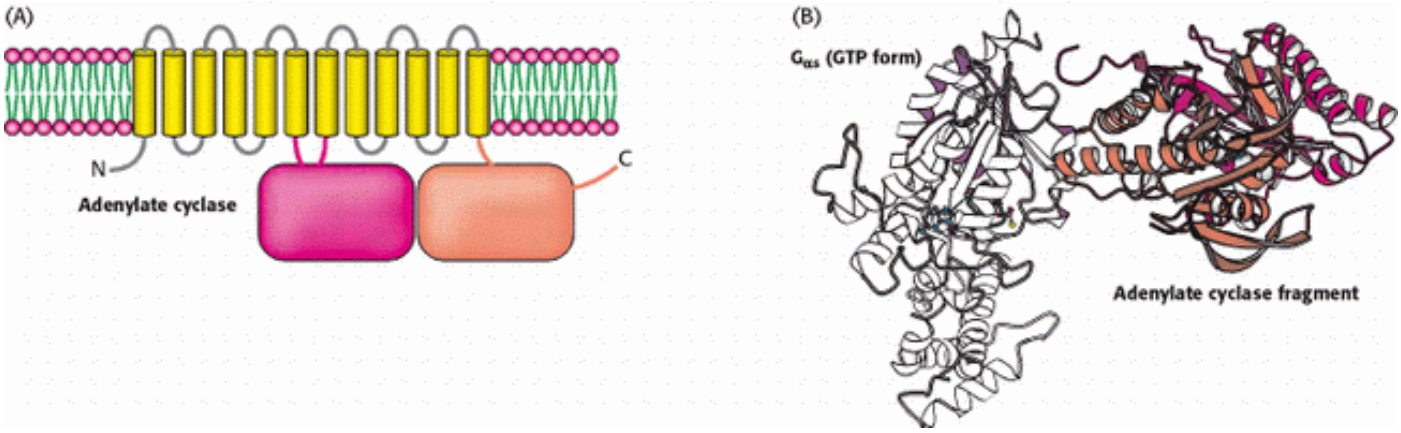


Figure 15.8. Adenylate Cyclase Is Activated by $G_{\alpha s}$. (A) Adenylate cyclase is an integral membrane protein with two large cytoplasmic domains that form the catalytic structure. (B) $G_{\alpha s}$ bound to GTP binds to the catalytic part of the cyclase, inducing a structural change that stimulates enzyme activity. The surface of $G_{\alpha s}$ that interacts with adenylate cyclase is the one that is exposed on release of $G_{\beta\gamma}$.

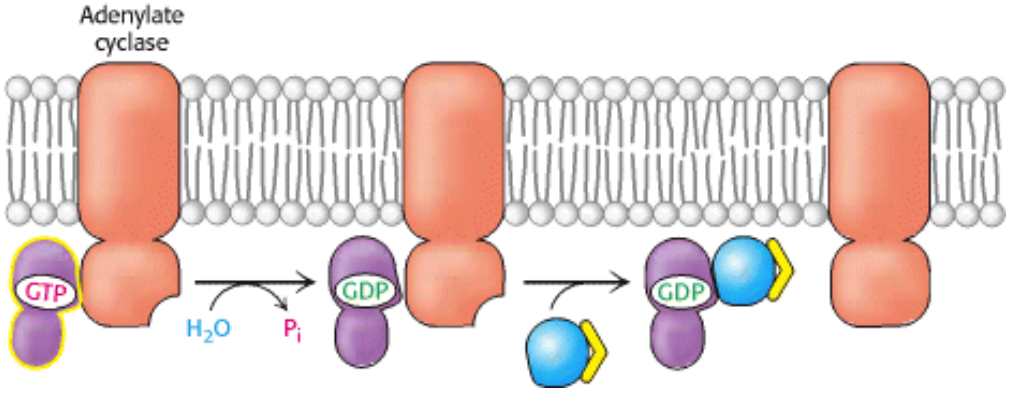


Figure 15.9. Resetting G_{α}. On hydrolysis of the bound GTP by the intrinsic GTPase activity of G_{α}, G_{α} reassociates with the $\beta\gamma$ subunits to form the heterotrimeric G protein, thereby terminating the activation of adenyl cyclase.

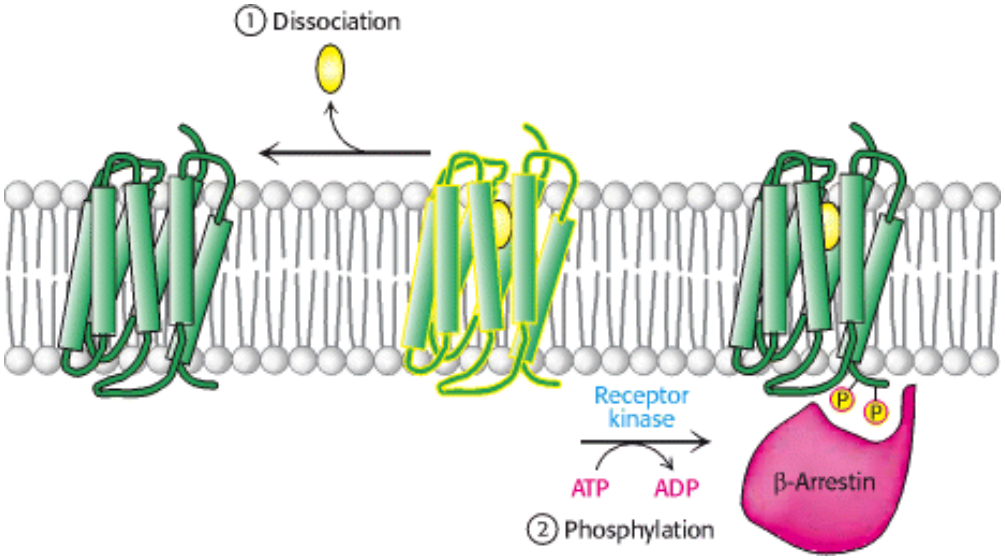
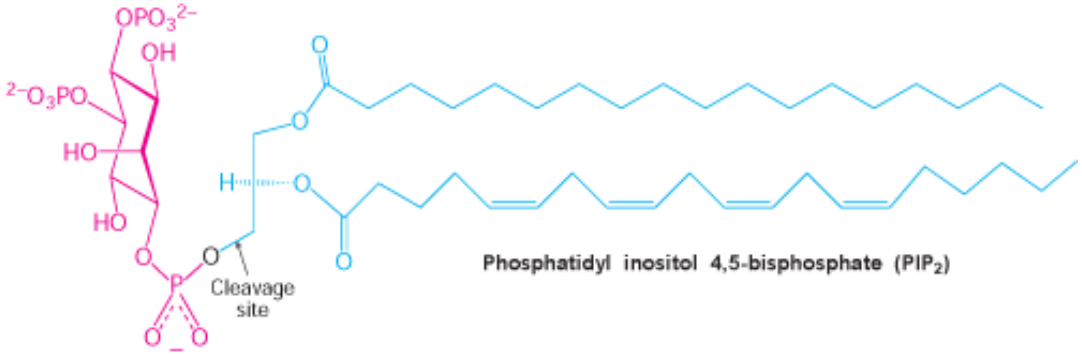


Figure 15.10. Signal Termination. Signal transduction by the 7TM receptor is halted (1) by dissociation of the signal molecule from the receptor and (2) by phosphorylation of the cytoplasmic C-terminal tail of the receptor and the

subsequent binding of β-arrestin.

15.2. The Hydrolysis of Phosphatidyl Inositol Bisphosphate by Phospholipase C Generates Two Messengers

Cyclic AMP is not the only second messenger employed by 7TM receptors and the G proteins. We turn now to another ubiquitous second-messenger cascade that is used by many hormones to evoke a variety of responses. The *phosphoinositide cascade*, like the adenylate cyclase cascade, converts extracellular signals into intracellular ones. The intracellular messengers formed by activation of this pathway arise from the cleavage *phosphatidyl inositol 4,5-bisphosphate* (PIP_2), a phospholipid present in cell membranes. The binding of a hormone such as vasopressin to a 7TM receptor leads to the activation of the β isoform of *phospholipase C*. The G_α protein that activates phospholipase C is called $G_{\alpha q}$. The activated enzyme then hydrolyzes the phosphodiester bond linking the phosphorylated inositol unit to the acylated glycerol moiety. The cleavage of PIP_2 produces two messengers: inositol 1,4,5-trisphosphate, a soluble molecule that can diffuse from the membrane, and diacylglycerol, which stays in the membrane.



Comparison of the amino acid sequences of different isoforms of phospholipase C as well as examination of the known three-dimensional structures of phospholipase components reveal an intriguing modular structure (Figure 15.11).

This analysis reveals the basis for both phospholipase enzymatic activity and its regulation by signal-transduction pathways. The catalytic core of these enzymes has an α β barrel structure similar to the catalytic core of triose phosphate isomerase and other enzymes (Section 16.1.4). This domain is flanked by domains that interact with membrane components. At the amino terminus is a pleckstrin homology (PH) domain. This ~120-residue domain binds a lipid head group such as that of PIP_2 (Figure 15.12). The PH domain is joined to the catalytic domain by a set of four EF-hand domains. Although EF-hand domains often take part in calcium-binding (Section 15.3.2), the EF-hand domains of phospholipase C lack many of the calcium-binding residues. On the carboxyl-terminal side of the catalytic domain is a C2 domain (for protein kinase *C* domain *2*). This ~130-residue domain is a member of the immunoglobulin domain superfamily (Chapter 33) and plays a role in binding phospholipid headgroups. Such interactions, often but not always, require the presence of bound calcium ions.

The binding of a G protein brings the enzyme into a catalytically active position. The β isoform of phospholipase C has an additional domain at its carboxyl terminus—a domain that interacts with the α subunit of G_q in its GTP form. Because this G protein is linked to the membrane by its fatty acid anchor, this interaction helps pull the β isoform of phospholipase to the membrane. This interaction acts in concert with the binding of the PH and C2 domains of phospholipase C to membrane components to bring the active site in the catalytic core into a position against a membrane surface that is favorable for cleavage of the phosphodiester bond of PIP_2 (Figure 15.13). Some of these interactions and the enzymatic reaction itself also depend on the presence of calcium ion. Phospholipase isoforms that lack the carboxyl-terminal regulatory domain do not respond to these signal-transduction pathways. The two products of the cleavage reaction, inositol 1,4,5-trisphosphate and diacylglycerol, each trigger additional steps in the signal-

transduction cascades.

15.2.1. Inositol 1,4,5-trisphosphate Opens Channels to Release Calcium Ions from Intracellular Stores

What are the biochemical effects of the second messenger inositol 1,4,5-trisphosphate? These effects were delineated by microinjecting IP_3 molecules into cells or by allowing IP_3 molecules to diffuse into cells whose plasma membranes had been made permeable. Michael Berridge and coworkers found that *IP_3 causes the rapid release of Ca^{2+} from intracellular stores—the endoplasmic reticulum and, in smooth muscle cells, the sarcoplasmic reticulum.* The elevated level of Ca^{2+} in the cytosol then triggers processes such as smooth muscle contraction, glycogen breakdown, and vesicle release. In *Xenopus* oocytes, the injection of IP_3 suffices to activate many of the early events of fertilization.

Inositol 1,4,5-trisphosphate is able to increase Ca^{2+} concentration by associating with a membrane protein called the *IP_3-gated channel* or *IP_3 receptor*. This receptor, which is composed of four large, identical subunits, forms an ion channel. The ion-conducting channel itself is likely similar to the structurally characterized K^+ channel (Section 13.5.6). At least three molecules of IP_3 must bind to sites on the cytosolic side of the membrane protein to open the channel and release Ca^{2+}. *The highly cooperative opening of calcium channels by nanomolar concentrations of IP_3 enables cells to detect and amplify very small changes in the concentration of this messenger.* The cooperativity of IP_3 binding and channel opening is another example of the role of allosteric interactions.

How is the IP_3-initiated signal turned off? *Inositol 1,4,5-trisphosphate is a short-lived messenger because it is rapidly converted into derivatives that do not open the channel* (Figure 15.14). Its lifetime in most cells is less than a few seconds. Inositol 1,4,5-trisphosphate can be degraded to inositol by the sequential action of phosphatases or it can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate, which is then converted into inositol by an alternative route. Lithium ion, widely used to treat bipolar affective disorder, may act by inhibiting the recycling of inositol 1,3,4-trisphosphate.

15.2.2. Diacylglycerol Activates Protein Kinase C, Which Phosphorylates Many Target Proteins

Diacylglycerol, the other molecule formed by the receptor-triggered hydrolysis of PIP_2, also is a second messenger and it, too, activates a wide array of targets. We will examine how diacylglycerol activates *protein kinase C (PKC)*, a protein kinase that phosphorylates serine and threonine residues in many target proteins. The amino acid sequences and the results of three-dimensional structural studies of protein kinase C isozymes reveal an elegant modular architecture (Figure 15.15). The α, β, and γ isozymes of protein kinase C have in common a structurally similar catalytic domain, homologous to that in PKA, at the carboxyl terminus. Adjacent to the catalytic domain is a C2 domain that is related to the C2 domain of phospholipase C and that interacts with membrane phospholipids. On the other side of the C2 domain is a pair of C1 domains, each organized around two bound zinc ions. These two domains, particularly the second (C1B) domain, bind diacylglycerol (Figure 15.16A). Finally, at the amino terminus is a sequence of the form -A-R-K-G-**A**-L-R-Q-K-. This sequence is striking because the consensus sequence for PKC substrates is X-R-X-X-(S,T)-Hyd-R-X in which Hyd refers to a large, hydrophobic residue. This sequence from PKC is referred to as a *pseudosubstrate sequence* because it resembles the substrate sequence except that it has an alanine residue in place of the serine or threonine residue; so it cannot be phosphorylated. Recall that an analogous pseudosubstrate sequence is present in the regulatory chain of PKA and that it prevents activity by occluding the active site in the R_2C_2 tetramer of PKA (Section 10.4.2). Similarly, the pseudosubstrate sequence of PKC binds to that enzyme's active site and prevents substrate binding.

With this structure in mind, we can now understand how PKC is activated on PIP_2 hydrolysis (Figure 15.16B). Before activation, PKC is free in solution. On PIP_2 hydrolysis in the membrane by phospholipase C, the C1B domain of PKC

binds to diacylglycerol. This binding and the interaction of the C2 domain with membrane phospholipids anchors the enzyme to the membrane. The interaction between the C2 domain and the phospholipids, especially phosphatidyl serine, requires calcium ions. The binding of the C1A domain to diacylglycerol pulls the pseudosubstrate out of the active site; the released pseudosubstrate, which is quite positively charged, probably interacts with the negatively charged membrane surface as well. The result of the conformational transitions is the binding of PKC to diacylglycerol-rich regions of the membrane in an active state, ready to phosphorylate serine and threonine residues in appropriate sequence contexts. Note that diacylglycerol and IP_3 work in tandem: IP_3 increases the Ca^{2+} concentration, and Ca^{2+} facilitates PKC activation as well as phospholipase C activity.

Diacylglycerol, like IP_3, acts transiently because it is rapidly metabolized. It can be phosphorylated to phosphatidate (Section 26.1) or it can be hydrolyzed to glycerol and its constituent fatty acids (Figure 15.17). Arachidonate, the C_{20} polyunsaturated fatty acid that usually occupies the position 2 on the glycerol moiety of PIP_2, is the precursor of a series of 20-carbon hormones, including the prostaglandins (Section 22.6.2). Thus, *the phosphoinositide pathway gives rise to many molecules that have signaling roles.*

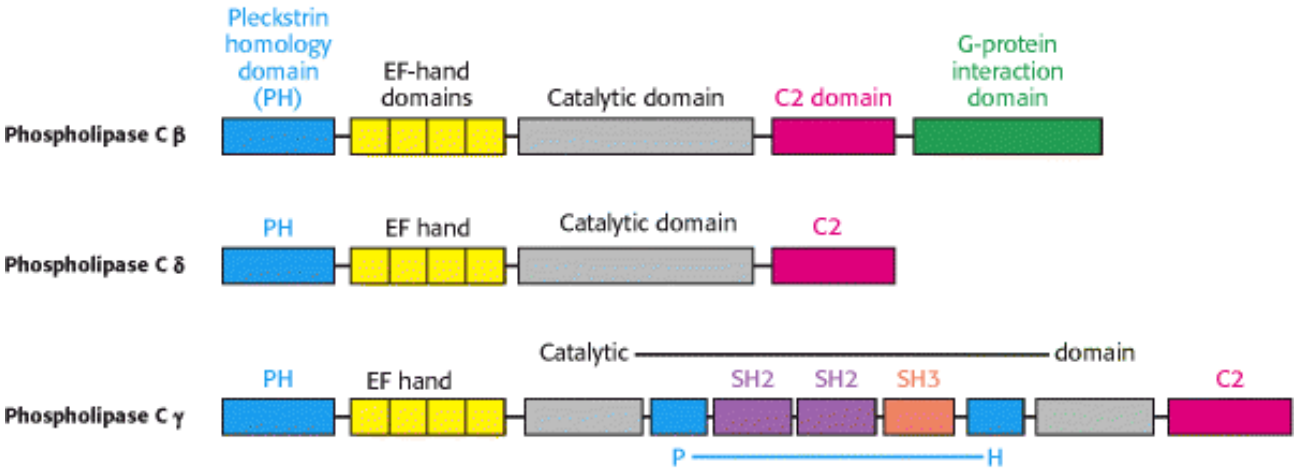


Figure 15.11. Modular Structure of Phospholipase C. The domain structures of three isoforms of phospholipase C reveal similarities and differences among the isoforms. Only the β isoform, with the G-protein-binding domain, can be stimulated directly by G proteins. For phospholipase Cγ, the insertion of two SH2 (Src homology 2) domains and one SH3 (Src homology 3) domain splits the catalytic domain and a PH domain into two parts.

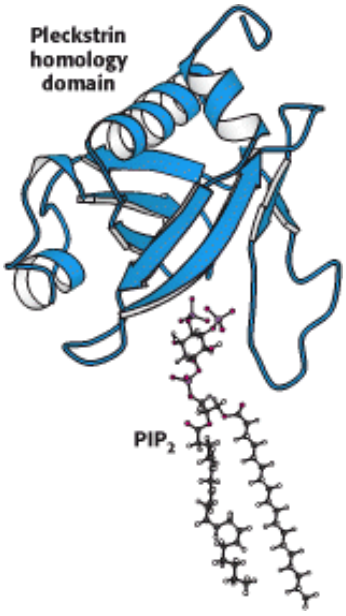


Figure 15.12. Pleckstrin Homology Domain. PH domains facilitate the binding of proteins to membrane lipids, particularly PIP_2. In regard to phospholipase C, the PH domains help to localize the enzyme near its substrate, PIP_2.

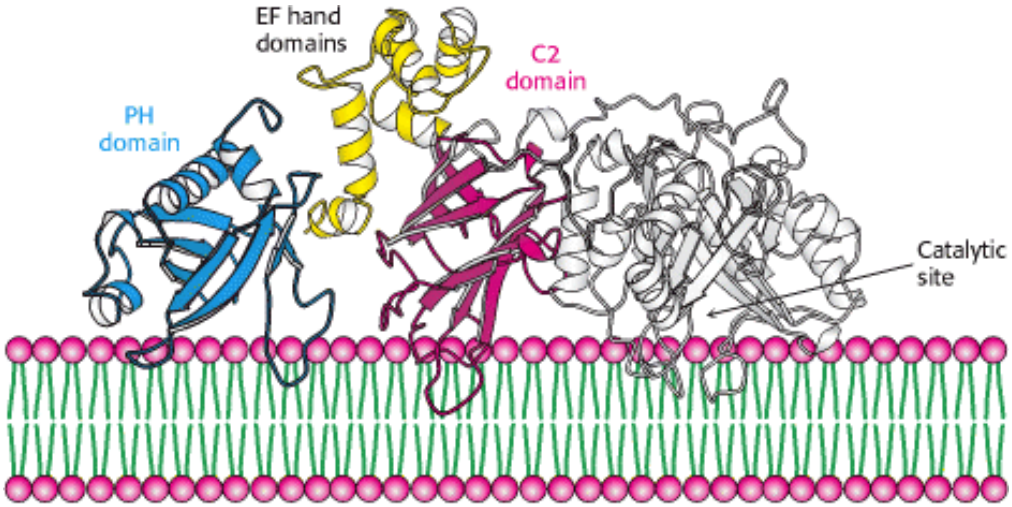


Figure 15.13. Phospholipase C Acts at the Membrane Surface. The PH and C2 domains of phospholipase help to position the enzyme's catalytic site for ready access to the phosphodiester bond of the membrane lipid substrate, PIP_2.

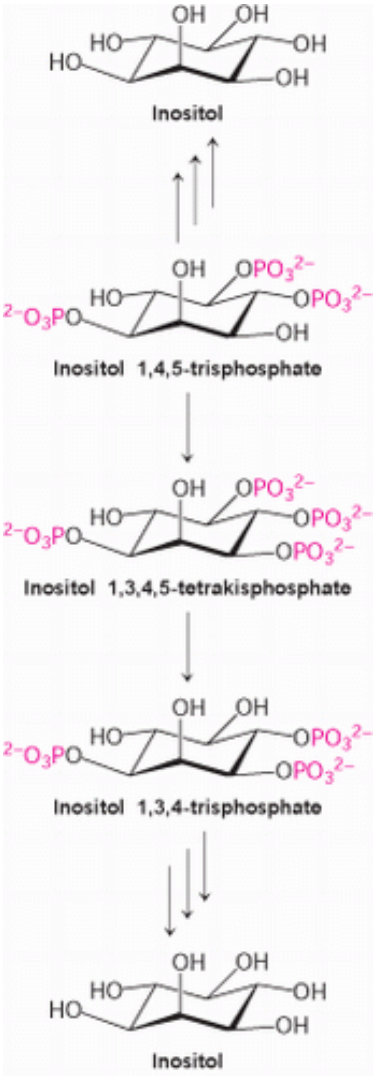


Figure 15.14. Metabolism of IP_3. The IP_3 signal is terminated by the metabolism of the compound into derivatives lacking second-messenger capabilities.

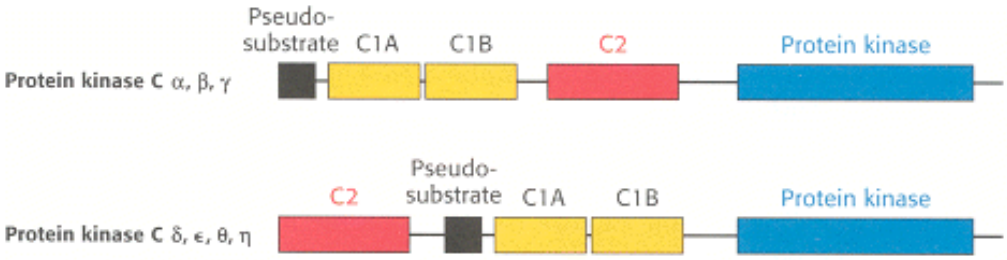


Figure 15.15. Modular Structure of Protein Kinase C. Seven isozymes of PKC can be divided into two classes on the basis of their domain organization.

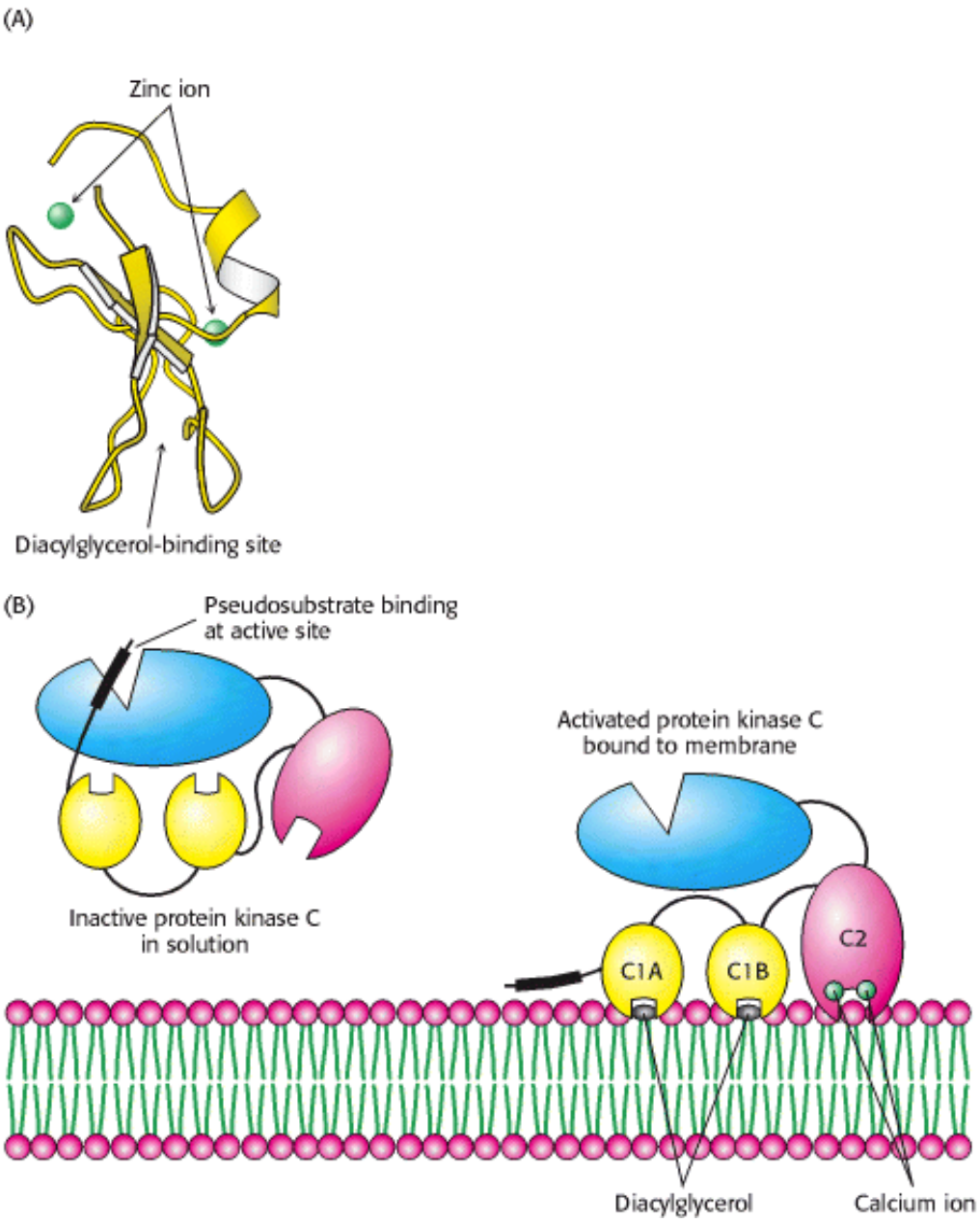


Figure 15.16. Protein Kinase C Activation. (A) The C1 domain of PKC, structurally organized around two bound zinc ions, binds diacylglycerol. (B) When the C1 domains bind to diacylglycerol in the membrane, the pseudosubstrate is pulled from the active site, permitting catalysis. Calcium-binding C2 domains help to localize PKC to the membrane.

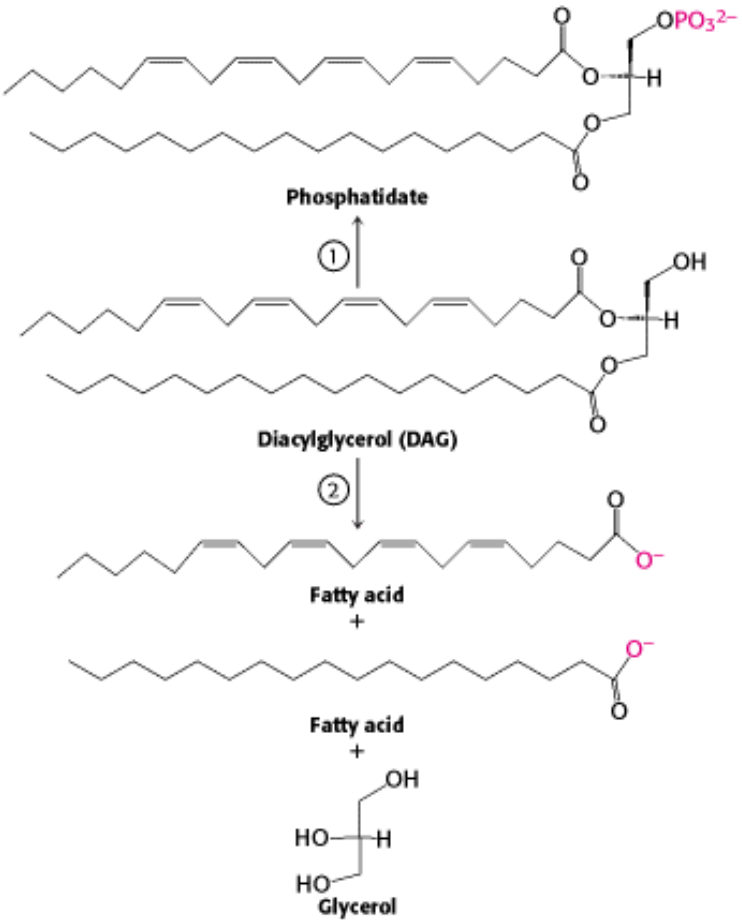


Figure 15.17. Metabolism of Diacylglycerol. Diacylglycerol may be (1) phosphorylated to phosphatidate or (2) hydrolyzed to glycerol and fatty acids.

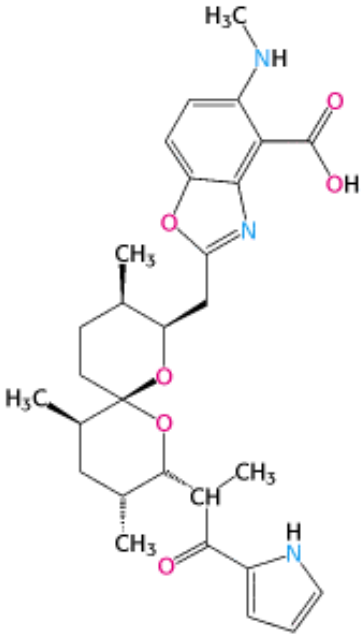
15.3. Calcium Ion Is a Ubiquitous Cytosolic Messenger

We have already seen that Ca^{2+} is an important component of one signal-transduction circuit, the phosphoinositide cascade. Indeed, Ca^{2+} is itself an intracellular messenger in many eukaryotic signal-transducing pathways. Why is this ion commonly found to mediate so many signaling processes? First, an apparent drawback is in fact an advantage: calcium complexes of phosphorylated and carboxylated compounds are often insoluble, but such compounds are fundamental to many biochemical processes in the cell. Consequently, the intracellular levels of Ca^{2+} must be kept low to prevent precipitation of these compounds. These low levels are maintained by transport systems for the extrusion of Ca^{2+}. In eukaryotic cells, two in particular—the Ca^{2+} ATPase (Section 13.2.1) and the sodium-calcium exchanger—are especially important. Because of their actions, the cytosolic level of Ca^{2+} in unexcited cells is typically 100 nM, several orders of magnitude lower than the concentration in the blood, which is approximately 5 mM. This steep concentration gradient presents cells with a matchless opportunity: *the cytosolic Ca^{2+} concentration can be abruptly raised for signaling purposes by transiently opening calcium channels in the plasma membrane or in an intracellular membrane.*

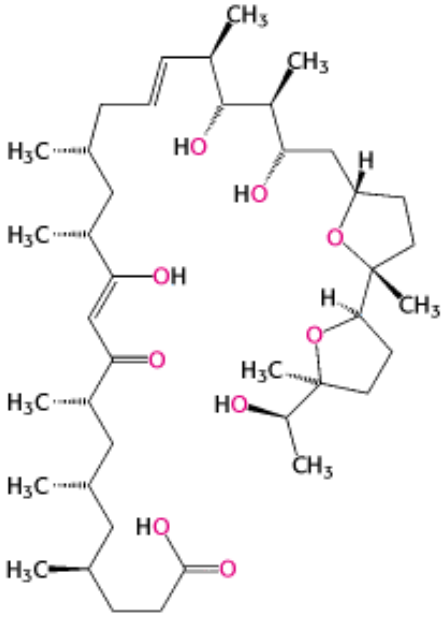
A second property of Ca^{2+} that makes it a highly suitable intracellular messenger is that it can bind tightly to proteins (Figure 15.18). Negatively charged oxygen atoms (from the side chains of glutamate and aspartate) and uncharged oxygen atoms (main-chain carbonyl groups and side-chain oxygen atoms from glutamine and asparagine) bind well to Ca^{2+}. *The capacity of Ca^{2+} to be coordinated to multiple ligands—from six to eight oxygen atoms—enables it to cross-link different segments of a protein and induce significant conformational changes.*

15.3.1. Ionophores Allow the Visualization of Changes in Calcium Concentration

Our understanding of the role of calcium in cellular processes has been greatly enhanced by the use of calcium-specific reagents. *Ionophores* such as *A23187* and *ionomycin* can traverse a lipid bilayer because they have a hydrophobic periphery.

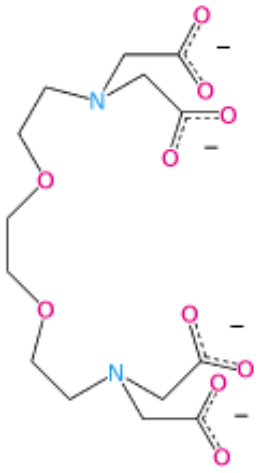


A23187

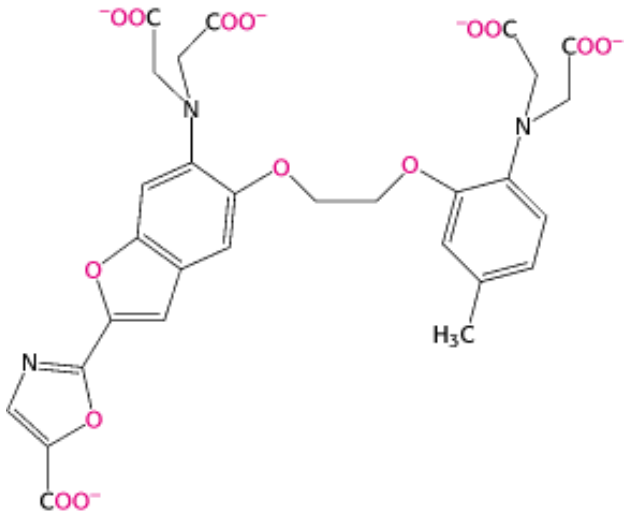


Ionomycin

They can be used to introduce Ca^{2+} into cells and organelles. Many physiological responses that are normally triggered by the binding of hormones to cell-surface receptors can also be elicited by using calcium ionophores to raise the cytosolic calcium level. Conversely, the concentration of unbound calcium in a cell can be made very low (nanomolar or less) by introducing a calcium-specific chelator such as EGTA. The concentration of free Ca^{2+} in intact cells can be monitored by using polycyclic chelators such as Fura-2.



EGTA
[Ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate]



Fura-2

The fluorescence properties of this and related indicators change markedly when Ca^{2+} is bound (Figure 15.19). These compounds can be introduced into cells to allow measurement of the available Ca^{2+} concentration in real time through the use of fluorescence microscopy. Such methods allow the direct detection of calcium fluxes and diffusion within living cells in response to the activation of specific signal-transduction pathways (Figure 15.20).

15.3.2. Calcium Activates the Regulatory Protein Calmodulin, Which Stimulates Many Enzymes and Transporters

Calmodulin (CaM), a 17-kd protein with four calcium-binding sites, serves as a calcium sensor in nearly all eukaryotic cells. *Calmodulin is activated by the binding of* Ca^{2+} *when the cytosolic calcium level is raised above about 500 nM.* Calmodulin is a member of the *EF-hand protein family.* The *EF hand* is a Ca^{2+}-binding motif that consists of a helix, a loop, and a second helix. This motif, originally discovered in the protein parvalbumin, was named the EF hand because helices designated E and F in parvalbumin form the calcium-binding motif and because the two helices are positioned like the forefinger and thumb of the right hand (Figure 15.21). Seven oxygen atoms are coordinated to each Ca^{2+}: six from the protein and one from a bound water molecule.

Let us consider how calmodulin changes conformation in response to Ca^{2+} binding and how these conformational changes enable the calmodulin to interact with other proteins (Figure 15.22). In the absence of bound Ca^{2+}, calmodulin consists of two domains, each consisting of a pair of EF-hand motifs joined by a flexible helix. Many of the residues that typically participate in calcium binding are on the surface of these domains, oriented in a manner inappropriate for Ca^{2+} binding. On the binding of one Ca^{2+} to each EF hand, these units change conformation: the Ca^{2+}-binding sites turn inward to bind the Ca^{2+}, moving hydrophobic residues from the inside to the outside of the domains. These conformational changes generate hydrophobic patches on the surface of each domain that are suitable for interacting with other proteins.

The Ca^{2+} *-calmodulin complex stimulates a wide array of enzymes, pumps, and other target proteins.* Two targets are especially noteworthy: one that propagates the signal and another that abrogates it. *Calmodulin-dependent protein kinases (CaM kinases)* phosphorylate many different proteins. These enzymes regulate fuel metabolism, ionic permeability, neurotransmitter synthesis, and neurotransmitter release. The binding of Ca^{2+}-calmodulin to CaM kinase activates the enzyme and enables it to phosphorylate target proteins. In addition, the activated enzyme phosphorylates itself and is thus partly active even after Ca^{2+}concentration falls and calmodulin is released from the kinase. In essence, autophosphorylation of CaM kinase is the memory of a previous calcium pulse. The plasma membrane *Ca* $^{2+}$ *-ATPase pump* is another important target of Ca^{2+}-calmodulin. Stimulation of the pump by Ca^{2+}-calmodulin drives the calcium level down to restore the lowcalcium basal state, thus helping to terminate the signal.

Are there structural features common to all calmodulin target proteins? Comparisons of the amino acid sequences of calmodulin-binding domains of target proteins suggests that *calmodulin recognizes positively charged, amphipathic α helices.* The results of structural studies fully support this conclusion and reveal the details of calmodulin-target interactions (Figure 15.23). The two domains of calmodulin surround the amphipathic helix and are linked to it by extensive hydrophobic and ionic interactions. The α helix linking the two EF hands folds back onto itself to facilitate binding to the target helix. How does this binding of calmodulin to its target lead to enzyme activation? In regard to CaM kinase I, calmodulin targets a peptide present at the carboxyl terminus of the kinase. This region interacts with a loop that is crucial for ATP binding and holds it in a conformation inappropriate for ATP binding. Calmodulin surrounds this peptide and extracts it, freeing the kinase active site to bind ATP and phosphorylate appropriate substrates.

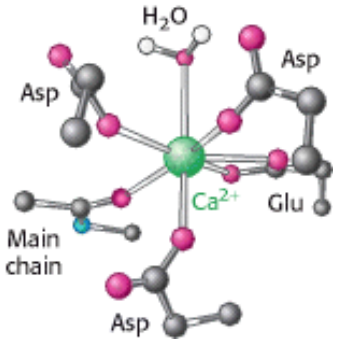


Figure 15.18. Mode of Binding of CA^{2+} to Calmodulin. Calcium is coordinated to six oxygen atoms from the protein and one (top) of water.

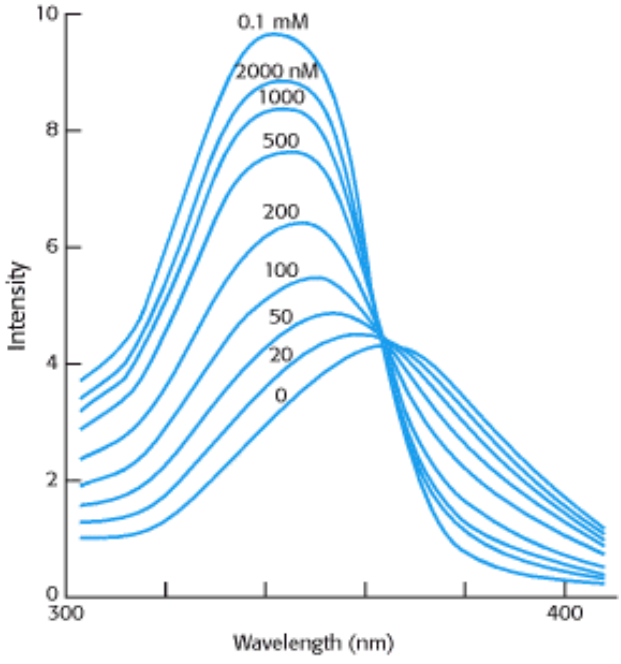


Figure 15.19. Calcium Indicator. The fluorescence spectra of the calcium-binding dye Fura-2 can be used to measure available calcium ion concentrations in solution and in cells. [After S. J. Lippard and J. M. Berg, *Principles of Bioinorganic Chemistry*. (University Science Books, 1994), p. 193.]

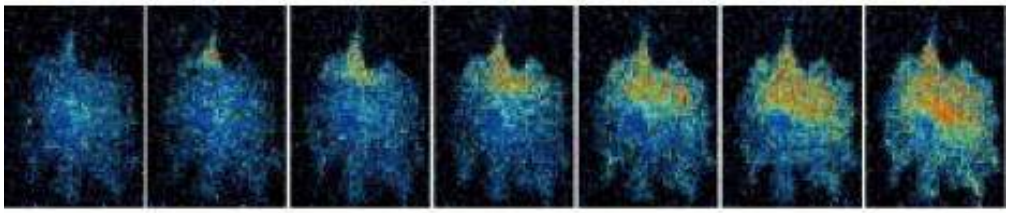


Figure 15.20. Calcium Imaging. A series of images shows Ca^{2+} spreading across a cell. These images were obtained through the use of a fluorescent calcium-binding dye. The images are false colored: red represents high Ca^{2+} concentrations and blue low Ca^{2+} concentrations. [Courtesy of Dr. Masashi Isshiki, Dept. of Nephrology, University of Tokyo, and Dr. G. W. Anderson, Dept. of Cell Biology, University of Texas Southwestern Medical School.]

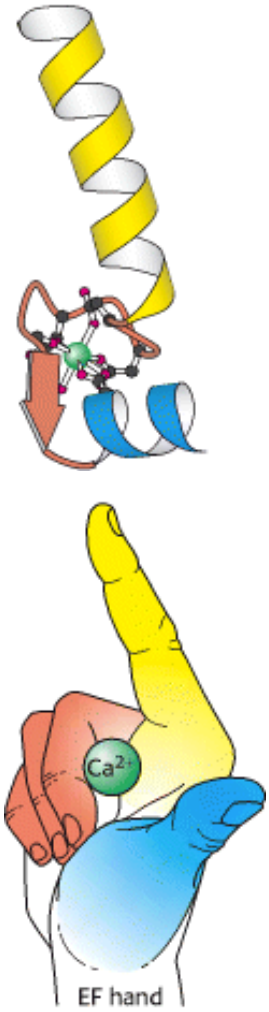


Figure 15.21. EF Hand. Formed by a helix-loop-helix unit, an EF hand is a binding site for calcium in many calcium sensing proteins. Here, the E helix is yellow, the F helix is blue, and calcium is represented by the green sphere.

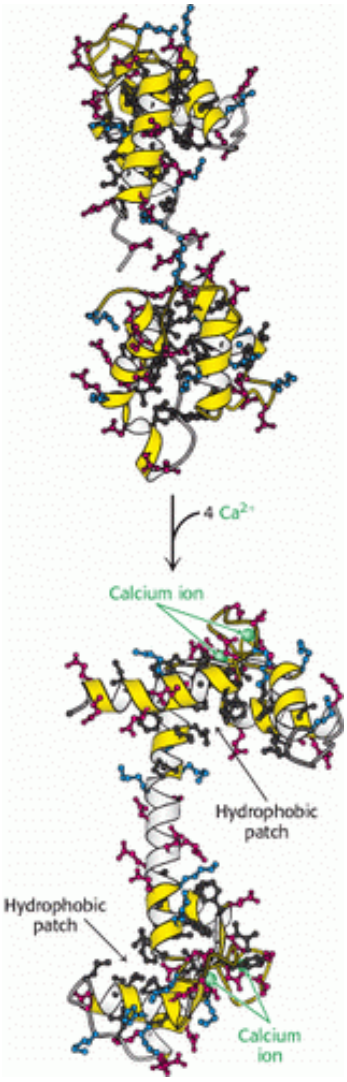


Figure 15.22. Conformational Changes in Calmodulin on Calcium Binding. In the absence of calcium (top), the EF hands have hydrophobic cores. On binding of a calcium ion (green sphere) to each EF hand, structural changes expose hydrophobic patches on the calmodulin surface. These patches serve as docking regions for target proteins. Acidic residues are shown in red, basic residues in blue, and hydrophobic residues in black. The central helix in calmodulin remains somewhat flexible, even in the calcium-bound state.

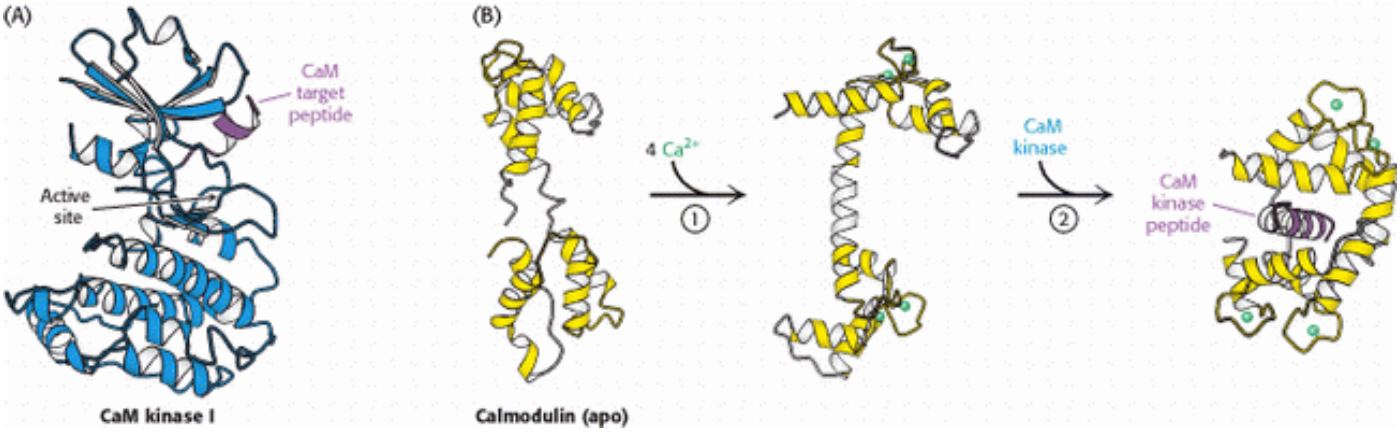


Figure 15.23. Calmodulin Binds to Amphipathic α Helices. (A) An amphipatic α helix (purple) in CaM kinase I is a

target for calmodulin. (B) After calcium binding (1), the two halves of calmodulin clamp down around the target helix (2), binding it through hydrophobic and ionic interactions. In CaM kinase I, this interaction extracts a C-terminal α helix, allowing the enzyme to adopt an active conformation.

15.4. Some Receptors Dimerize in Response to Ligand Binding and Signal by Cross-phosphorylation

The 7TM receptors initiate signal-transduction pathways through changes in tertiary structure that are induced by ligand binding. A fundamentally different mechanism is utilized by a number of other classes of receptors. For these receptors, ligand binding leads to changes in quaternary structures—specifically, the formation of receptor dimers. As we shall see, receptor dimerization is crucial because protein kinase domains associated with the intracellular domains of the receptors are brought together in such a way that they can phosphorylate one another. Such *cross-phosphorylation* initiates further signaling.

We consider human growth hormone and its receptor as our first example. Growth hormone is a monomeric protein of 217 amino acids that forms a compact four-helix bundle structure (Figure 15.24). The growth-hormone receptor comprises 638 amino acids, divided into an extracellular domain of 250 amino acids, a single membrane-spanning helix, and an intracellular domain of 350 amino acids. In the absence of bound hormone, the receptor is present as a monomer. Growth hormone binds to the extracellular domain of the receptor. Remarkably, each monomeric hormone binds to two receptor molecules, thus promoting the formation of a dimer of the receptor (Figure 15.25). The binding between the hormone and the receptors is highly cooperative; once a hormone has bound to a single receptor molecule, the binding of the second receptor is highly favored.

This process takes place outside the cell. Dimerization of the extracellular domains of the receptor brings together the intracellular domains as well. Associated with each intracellular domain is a molecule of a protein kinase termed *Janus kinase 2 (JAK2)* in an unactivated form. Janus kinases have modular structures consisting of four previously described domains (Figure 15.26).

The carboxyl terminus is a protein kinase domain; its amino acid sequence and known biochemical properties suggest that this domain functions as a tyrosine kinase. Adjacent to this domain is a second region that is clearly homologous to a protein kinase, although several key residues have been changed and the biochemical activity of this domain is not well established. Indeed, this pair of kinase-like domains accounts for the name of these proteins; Janus is the two-headed Roman god of gateways. At the amino terminus is a 300-amino-acid domain, called the ERM domain, that helps anchor JAK2 to membranes. In between this domain and the kinase domains is an *SH2 domain* (SH for Src homology; Section 15.4). SH2 domains are 100-amino-acid domains that bind peptides containing phosphotyrosine (Figure 15.27). The domains bind phosphotyrosine through interactions with conserved arginine residues among other residues.

Structural Insights, SH2 Domains: An Example of Modular Regulatory Domains, takes a close look at phosphotyrosine-SH2 domain interactions and the diverse ways they can affect protein function.

Dimerization of the growth-hormone receptors brings together the JAK2 proteins associated with each intracellular domain, apparently delivering a key loop (termed the *activation loop*) of one kinase domain into the active site of the kinase bound to the other receptor, which results in cross-phosphorylation (Figure 15.28). How phosphorylation of JAK2 leads to its activation has not been directly established. However, the results of studies of other kinases reveal that the activation loop is in a conformation unsuitable for catalysis in the unphosphorylated form but changes to an active conformation when phosphoryl groups are added to key sites (Figure 15.29).

When activated by cross-phosphorylation, JAK2 can phosphorylate other substrates. In the present case, at least two important proteins are phosphorylated—a regulator of gene expression called STAT5 (STAT for signal *t*ransducers and

*a*ctivators of *t*ranscription) and the growth-hormone receptor itself. What are the consequences of the phosphorylation of these two proteins? STAT5 is phosphorylated on a tyrosine residue near the carboxyl terminus of the protein. The phosphotyrosine residue binds to an SH2 domain of another STAT5 molecule. Reciprocal interactions lead to the formation of a stable STAT dimer (Figure 15.30). The dimerized STAT protein, which has a much greater affinity for specific binding sites on DNA than does a monomeric protein, moves to the nucleus, where it binds to the DNA binding sites to regulate gene expression. The phosphorylation of the growth-hormone receptor may have several consequences. First, the phosphorylated receptor may serve as a docking site for JAK2 through its SH2 domain. Second, other proteins may associate with the phosphorylated receptor, participating in other signaling pathways.

15.4.1. Some Receptors Contain Tyrosine Kinase Domains Within Their Covalent Structures

Growth factors such as *insulin, epidermal growth factor (EGF),* and *platelet-derived growth factor* bind to the extracellular domains of transmembrane receptors that have tyrosine kinase domains present within their intracellular domains. For these proteins, which are found in multicellular organisms but not in yeast, genes encoding extracellular domains and the signaling kinases fused in the course of evolution. These *receptor tyrosine kinases (RTKs)* signal by mechanisms quite similar to those discussed for the pathway initiated by the growth-hormone receptor.

Consider, for example, *epidermal growth factor,* a 6-kd polypeptide that stimulates the growth of epidermal and epithelial cells (Figure 15.31). This 53-residue growth factor is produced by the cleavage of an *EGF precursor,* a large transmembrane protein. Such processing, which is common for growth factors and hormones, is reminiscent of the processing of zymogens into active enzymes (Section 10.5). The first step in the signal-transduction pathway is the binding of EGF to the *epidermal growth factor receptor,* a single polypeptide chain consisting of 1186 residues. The receptor tyrosine kinase is monomeric and enzymatically inactive in the absence of the growth factor. *The binding of EGF to the extracellular domain causes the receptor to dimerize and undergo cross-phosphorylation and activation.*

The insulin receptor is a disulfide-bonded dimer of $\alpha\,\beta$ pairs even when insulin is not bound. Nevertheless, insulin is still required for the activation of the kinase, demonstrating that dimerization is necessary but not sufficient for activation. The binding of the growth factor must convert the subunits of the dimer into a conformation that brings appropriate tyrosine residues from one chain into the active site of the other chain so that cross-phosphorylation can take place.

An elegant experiment demonstrated the commonality of the receptor tyrosine kinase signaling mechanism. The EGF receptor and the insulin receptor both contain intrinsic tyrosine kinases. Do these receptors transfer information across the membrane in the same way? This question was answered by synthesizing a gene that encoded a *chimeric receptor*—the extracellular part came from the insulin receptor, and the membrane-spanning and cytosolic parts came from the EGF receptor. The striking result was that the binding of insulin induced tyrosine kinase activity, as evidenced by rapid autophosphorylation. Hence, *the insulin receptor and the EGF receptor employ a common mechanism of signal transmission across the plasma membrane.*

How is the signal transferred beyond the receptor tyrosine kinase? We have seen that activated tyrosine kinases can phosphorylate other proteins and that phosphotyrosines on the phosphorylated receptors can act as docking sites for SH2 domains on other proteins. A key *adaptor protein* links the phosphorylation of the EGF receptor to the stimulation of cell growth through a chain of protein phosphorylations (Figure 15.32). On phosphorylation of the receptor, the SH2 domain of the adaptor protein Grb-2 binds to the phosphotyrosine residues of the receptor tyrosine kinase. Grb-2 then recruits a protein called Sos, which interacts with Grb-2 through two *SH3 domains,* domains that bind proline-rich stretches of polypeptide and, like SH2 domains, are recurring domains that mediate protein-protein interactions. Sos, in turn, binds to and activates Ras, a very prominent signal-transduction component that we will consider in Section 15.4.2. Finally, Ras, in its activated form, binds to other components of the molecular circuitry leading to the activation of the specific serine-threonine protein kinases that phosphorylate specific targets that promote cell growth. We see here another example of how a signal-transduction pathway is constructed. Specific protein-protein interactions (through SH2, SH3, and other domains not considered here) link the original ligand-binding event to the final result—stimulation of cell growth.

15.4.2. Ras, Another Class of Signaling G Protein

We now turn our attention to another important family of signal proteins, the *small G proteins*, or small GTPases. This large superfamily of proteins—grouped into subfamilies called Ras, Rho, Arf, Rab, and Ran—plays a major role in a host of cell functions including growth, differentiation, cell motility, cytokinesis, and transport of materials throughout the cell (Table 15.3). Like their relatives the heterotrimeric G proteins (Section 15.1.2), the small G proteins cycle between an active GTP-bound form and an inactive GDP-bound form. They differ from the heterotrimeric G proteins in being smaller (20–25 kd versus 30–35 kd) and monomeric. Nonetheless, the two families are related by divergent evolution, and small G proteins have many key mechanistic and structural motifs in common with the G_{α} subunit of the heterotrimeric G proteins.

In their activated GTP-bound form, small G proteins such as Ras stimulate cell growth and differentiation. Recall that Sos is the immediate upstream link to Ras in the circuit conveying the EGF signal. How does Sos activate Ras? Sos binds to Ras, reaches into the nucleotide-binding pocket, and opens it up, allowing GDP to escape and GTP to enter in its place (Figure 15.33). This process is presumably analogous to the stimulation of nucleotide exchange in heterotrimeric G proteins by activated 7TM receptors, a process for which structural details are not yet available. Sos is referred to as a *guaninenucleotide exchange factor (GEF)*. Thus, the binding of EGF to the EGF receptor leads to the conversion of Ras into its GTP form through the intermediacy of Grb-2 and Sos (Figure 15.34).

Like the G_{α} protein, Ras possesses an intrinsic GTPase activity, which serves to terminate the signal and return the system to the inactive state. This activity is slow but is augmented by helper proteins termed *GTPase-activating proteins (GAPs)*. The guanine-nucleotide exchange factors and the GTPase-activating proteins allow the G-protein cycle to proceed with rates appropriate for a balanced level of downstream signaling.

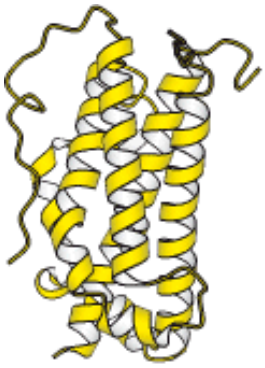


Figure 15.24. Human Growth Hormone Structure. Human growth hormone forms a four-helix bundle.

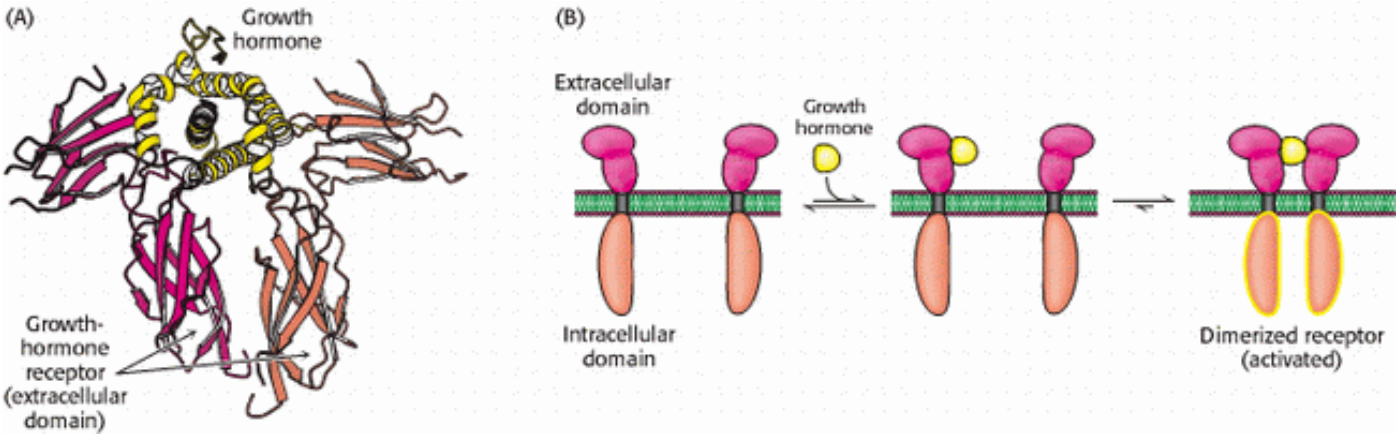


Figure 15.25. Binding of Growth Hormone Leads to Receptor Dimerization. (A) A single growth-hormone molecule (yellow) interacts with the extracellular domain of two receptors (red and orange). (B) The binding of one hormone molecule to two receptors leads to the formation of a receptor dimer. Dimerization is a key step in this signal-transduction pathway.

Figure 15.26. Janus Kinase Domain Structure. A Janus kinase (JAK) includes four recognized domains: an ERM domain that favors interactions with membranes, an SH2 domain that binds phosphotyrosine-containing peptides, and two domains homologous to protein kinases. Only the second protein kinase domain appears to be enzymatically functional.

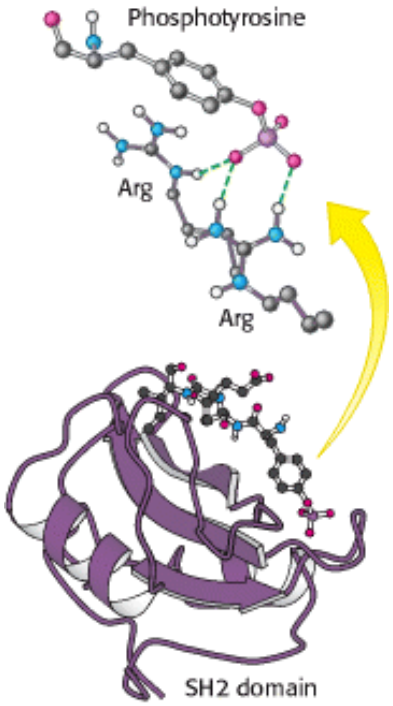


Figure 15.27. Recognition of Phosphotyrosine by SH2 Domains. The structure of an SH2 domain (purple) bound to a phosphotyrosine-containing peptide. The hydrogen-bonding interactions between the phosphotyrosine residue and two arginine residues are shown; interactions with other residues are omitted for clarity.

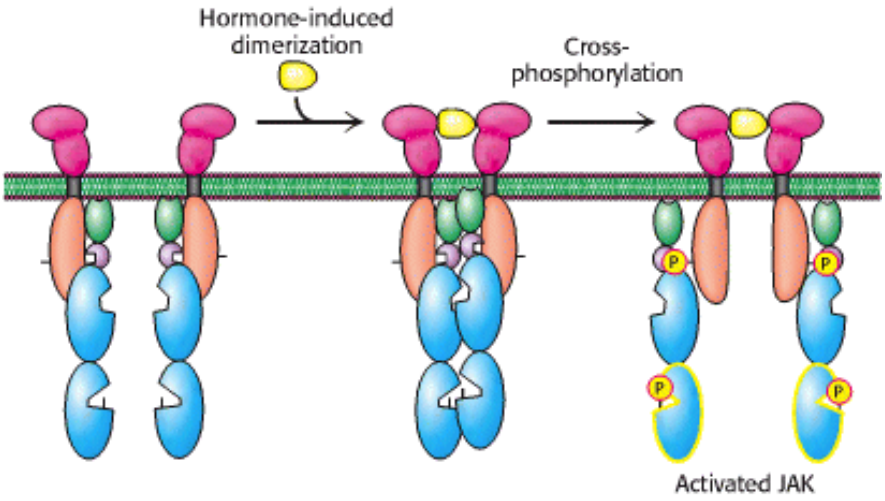


Figure 15.28. Cross-Phosphorylation of Jaks Induced by Receptor Dimerization. The binding of growth hormone leads to receptor dimerization, which brings two JAKs together in such a way that each phosphorylates key residues on the other. The activated JAKs remain bound to the receptor.

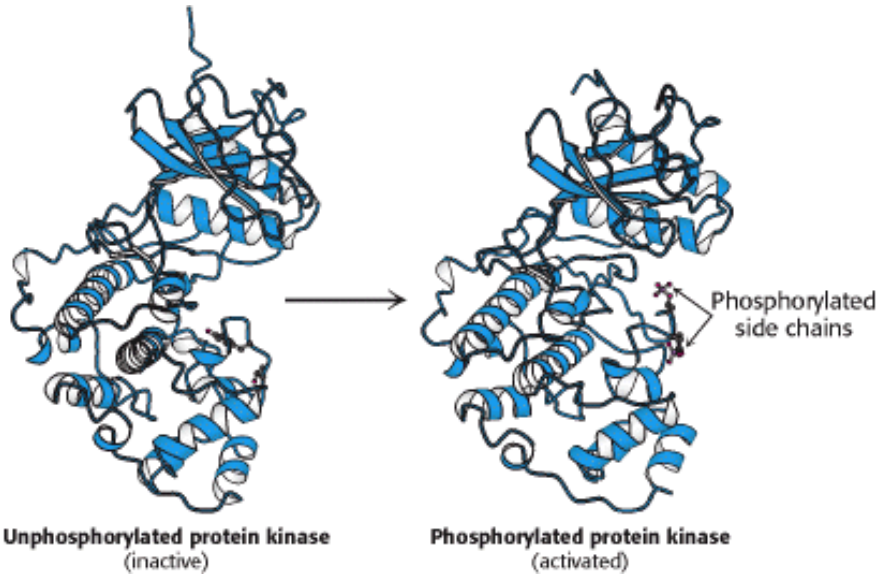


Figure 15.29. Activation of a Protein Kinase by Phosphorylation. In the unphosphorylated state, a key loop is in a conformation unsuitable for catalysis. Phosphorylation (at two sites in the case shown) stabilizes an active conformation.

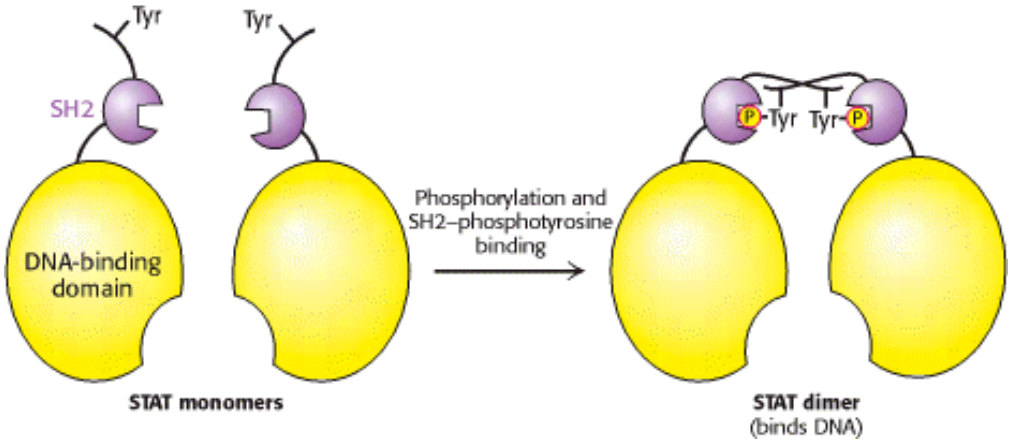


Figure 15.30. Phosphorylation-Induced Dimerization of STAT Proteins. The phosphorylation of a key tyrosine residue on each STAT protein leads to an interaction between the phosphotyrosine and an SH2 domain on another STAT monomer. The STAT dimer produced by these reciprocal interactions has a high affinity for specific DNA sequences and is able to alter gene expression after binding to DNA.

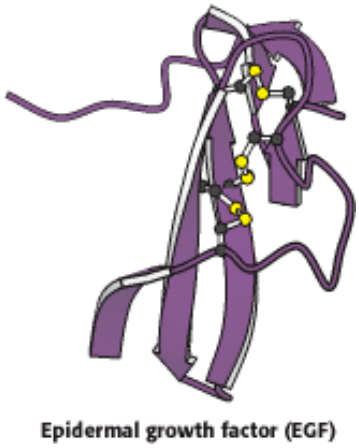


Figure 15.31. Structure of Epidermal Growth Factor. This protein growth factor is stabilized by three disulfide bonds.

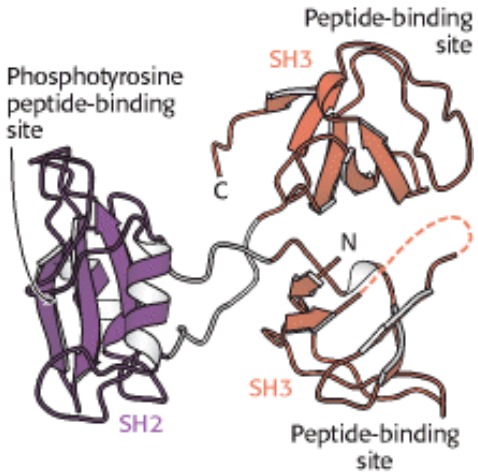


Figure 15.32. Structure of Grb-2, an Adaptor Protein. Grb-2 consists of two SH3 domains and a central SH2 domain. The SH2 domain binds to phosphotyrosine resides on an activated receptor while the SH3 domains bind proline-rich regions on other proteins such as Sos.

Table 15.3. Ras superfamily of GTPases

Subfamily	Function
Ras	Regulates cell growth through serine-threonine protein kinases
Rho	Reorganizes cytoskeleton through serine-threonine protein kinases
Arf	Activates the ADP-ribosyltransferase of the cholera toxin A subunit; regulates vesicular trafficking pathways; activates phospholipase D
Rab	Plays a key role in secretory and endocytotic pathways
Ran	Functions in the transport of RNA and protein into and out of the nucleus

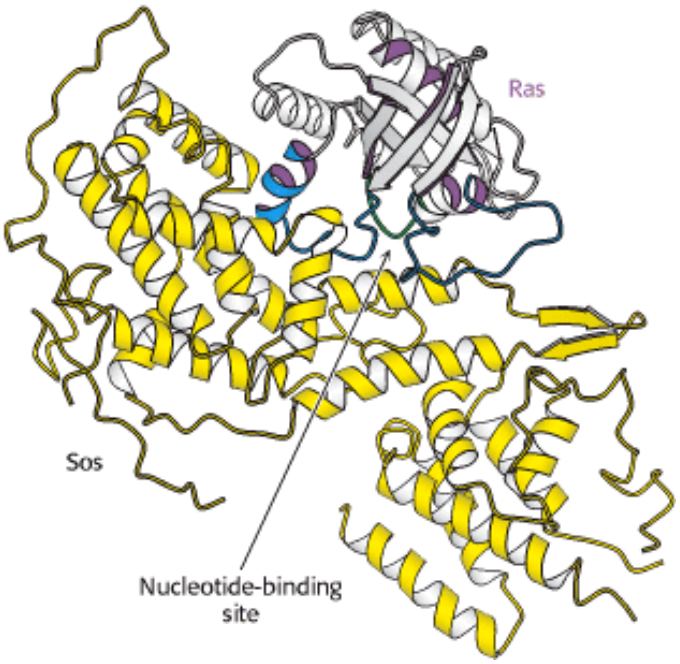


Figure 15.33. Structure of Sos, a Guanine-Nucleotide Exchange Factor. Sos (yellow) binds to Ras and opens up its nucleotide-binding site, allowing GDP to escape and GTP to bind. In the GTP-bound form, Ras can bind to and activate other proteins, including protein kinases.

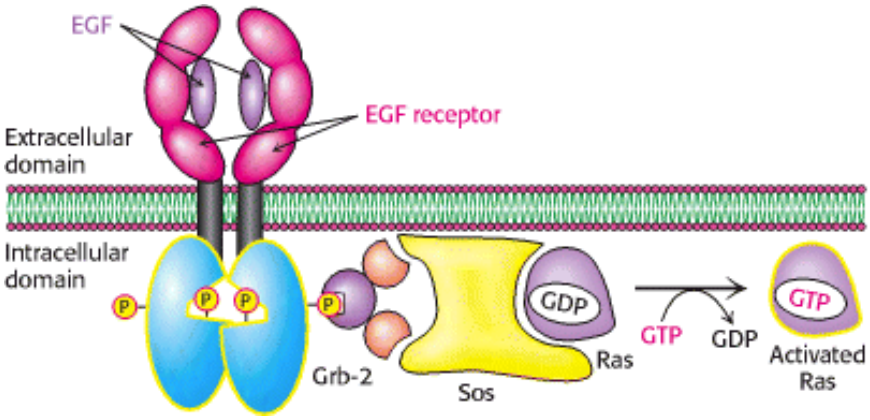


Figure 15.34. Egf Signaling Pathway. The binding of epidermal growth factor (EGF) to its receptor leads to cross-phosphorylation of the receptor. The phosphorylated receptor binds Grb-2, which, in turn, binds Sos. Sos stimulates the exchange of GTP for GDP in Ras. Activated Ras binds to and stimulates protein kinases (not shown).

15.5. Defects in Signaling Pathways Can Lead to Cancer and Other Diseases

In light of their complexity, it comes as no surprise that signal-transduction pathways occasionally fail, leading to pathological or disease states. Cancer, a set of diseases characterized by uncontrolled or inappropriate cell growth, is strongly associated with defects in signal-transduction proteins. Indeed, the study of cancer, particularly cancer caused by certain viruses, has contributed greatly to our understanding of signal-transduction proteins and pathways.

For example, Rous sarcoma virus is a retrovirus that causes sarcoma (a cancer of tissues of mesodermal origin such as muscle or connective tissue) in chickens. In addition to the genes necessary for viral replication, this virus carries a gene termed v-*src*. The v-*src* gene is an *oncogene*; it leads to the transformation of susceptible cell types. The protein encoded by v-*src* is a protein tyrosine kinase that includes SH2 and SH3 domains (Figure 15.35). Indeed, the names of these domains derive from the fact that they are *S*rc *h*omology domains. The v-Src protein is similar in amino acid sequence to a protein normally found in chicken muscle cells referred to as c-Src (for cellular Src). The c-*src* gene does not induce cell transformation and is termed a *proto-oncogene.* The protein that it encodes is a signal-transduction protein that regulates cell growth. As we shall see, small differences in the amino acid sequences between the proteins encoded by the proto-oncogene and the oncogene are responsible for the oncogene product being trapped in the "on" position.

An examination of the structure of c-Src in an inactive conformation reveals an intricate relation between the three major domains. The SH3 domain lies nearest the amino terminus, followed by the SH2 domain and then the kinase domain. There is also an extended carboxyl-terminal stretch that includes a phosphotyrosine residue. The phosphotyrosine residue is bound within the SH2 domain, whereas the linker between the SH2 domain and the kinase domain is bound by the SH3 domain. These interactions hold the kinase domain in an inactive conformation. The Src protein in this form can be activated by three distinct processes (Figure 15.36): (1) the phosphotyrosine residue bound in the SH2 pocket can be displaced by another phosphotyrosine-containing polypeptide with a higher affinity for this SH2 domain, (2) the phosphoryl group on the tyrosine residue can be removed by a phosphatase, and (3) the linker can be displaced from the SH3 domain by a polypeptide with a higher affinity for this SH3 domain. Thus, Src responds to the presence of one of a set of distinct signals. The amino acid sequence of the viral oncogene is more than 90% identical with its cellular counterpart. Why does it have such a different biological activity? The C-terminal 19 amino acids of c-Src are replaced by a completely different stretch of 11 amino acids, and this region lacks the key tyrosine residue that is phosphorylated in inactive c-Src. Since the discovery of Src, many other mutated protein kinases have been identified as oncogenes.

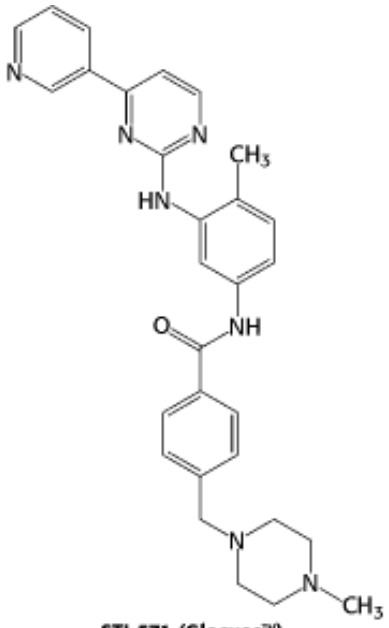
How did the Rous sarcoma virus acquire the mutated version of *src*? In an infection, a viral genome may pick up a gene from its host in such a way that the region encoding the last few amino acids is missing. Such a modified gene may have given the Rous sarcoma virus a selective advantage because it will have favored viral growth when introduced with the virus into a host cell.

Impaired GTPase activity in a regulatory protein also can lead to cancer. Indeed, *ras* is one of the genes most commonly mutated in human tumors. Mammalian cells contain three 21-kd Ras proteins (H-, K-, and N-Ras) that cycle between GTP and GDP forms. The most common mutations in tumors lead to a loss of the ability to hydrolyze GTP. Thus, the Ras protein is trapped in the "on" position and continues to stimulate cell growth.

15.5.1. Protein Kinase Inhibitors May Be Effective Anticancer Drugs

The widespread occurrence of over active protein kinases in cancer cells suggests that molecules that inhibit these enzymes might act as antitumor agents. Recent results have dramatically supported this concept. More than 90% of patients with chronic myologenous leukemia (CML) show a specific chromosomal defect in affected cells (Figure 15.37). The translocation of genetic material between chromosomes 9 and 22 causes the c-*abl* gene, which encodes a tyrosine kinase, to be inserted into the *bcr* gene on chromosome 22. The result is the production of a fusion protein called

Bcr-Abl that consists primarily of sequences for the c-Abl kinase. However, the *bcr-abl* gene is not regulated appropriately; it is expressed at higher levels than the gene encoding the normal c-Abl kinase. In addition, the Bcr-Abl protein may have regulatory properties that are subtly different from those of the c-Abl kinase itself. Thus, leukemia cells express a unique target for chemotherapy. Recent clinical trials of a specific inhibitor of the Bcr-Abl kinase have shown dramatic results; more than 90% of patients responded well to the treatment. This approach to cancer chemotherapy is fundamentally distinct from most approaches, which target cancer cells solely on the basis of their rapid growth, leading to side effects because normal rapidly growing cells also are affected. *Thus, our understanding of signal-transduction pathways is leading to conceptually new disease treatments.*



STI-571 (Gleevec™)
(Bcr-Abl kinase inhibitor)

15.5.2. Cholera and Whooping Cough Are Due to Altered G-Protein Activity

We consider here some pathologies of the G-protein-dependent signal pathways. Let us first consider the mechanism of action of the cholera toxin, secreted by the intestinal bacterium *Vibrio cholera.* Cholera is an acute diarrheal disease that can be life threatening. It causes voluminous secretion of electrolytes and fluids from the intestines of infected persons. The cholera toxin, *choleragen,* is a protein composed of two functional units—a B subunit that binds to G_{M1} gangliosides of the intestinal epithelium and a catalytic A subunit that enters the cell. The A subunit catalyzes the covalent modification of a G_{α^s} protein: the α subunit is modified by the attachment of an ADP-ribose to an arginine residue. This modification stabilizes the GTP-bound form of G_{α^s}, trapping the molecule in the active conformation. The active G protein, in turn, continuously activates protein kinase A. PKA opens a chloride channel (a CFTR channel; Section 13.3) and inhibits the Na^+-H^+ exchanger by phosphorylation. The net result of the phosphorylation of these channels is an excessive loss of NaCl and the loss of large amounts of water into the intestine. Patients suffering from cholera for 4 to 6 days may pass as much as twice their body weight in fluid. Treatment consists of rehydration with a glucose-electrolyte solution.

Whereas cholera is a result of a G protein trapped in the active conformation, causing the signal-transduction pathway to be perpetually stimulated, pertussis, or whooping cough, is a result of the opposite situation. Pertussis toxin also adds an ADP-ribose moiety,— in this case, to a G_{α^i} protein, a G_{α} protein that inhibits adenyl cyclase, closes Ca^{2+} channels, and opens K^+ channels. The effect of this modification, however, is to lower the G protein's affinity for GTP, effectively trapping it in the "off" conformation. The pulmonary symptoms have not yet been traced to a particular target of the G_{α^i} protein. Pertussis toxin is secreted by *Bordetella pertussis,* the bacterium responsible for whooping cough.

Cholera and pertussis are but two examples of diseases caused by defects in G proteins. Table 15.4 lists others. In light of the fact that G proteins relay signals for more than 500 receptors, it is likely that this list will continue to grow.

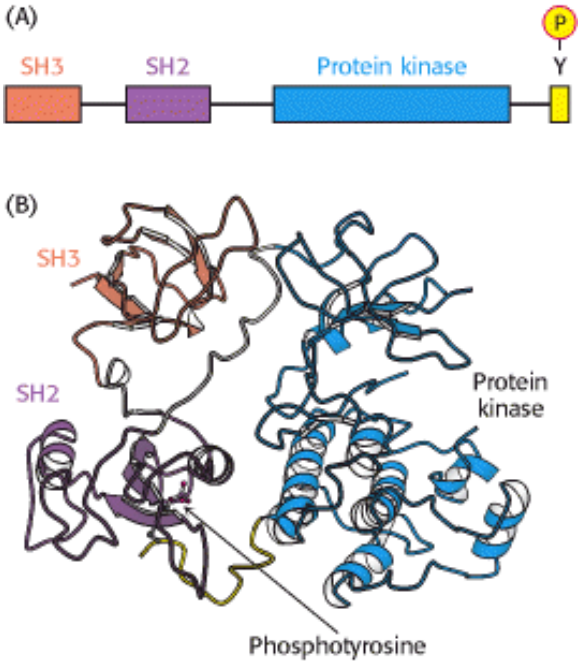


Figure 15.35. Src Structure. (A) Cellular Src includes an SH3 domain, an SH2 domain, a protein kinase domain, and a carboxyl-terminal tail that includes a key tyrosine residue. (B) Structure of c-Src in an inactivated form with the key tyrosine residue phosphorylated. The phosphotyrosine residue is bound in the SH2 domain; the linker between the SH2 domain and the protein kinase domain is bound by the SH3 domain. These interactions hold the kinase domain in an inactive conformation.

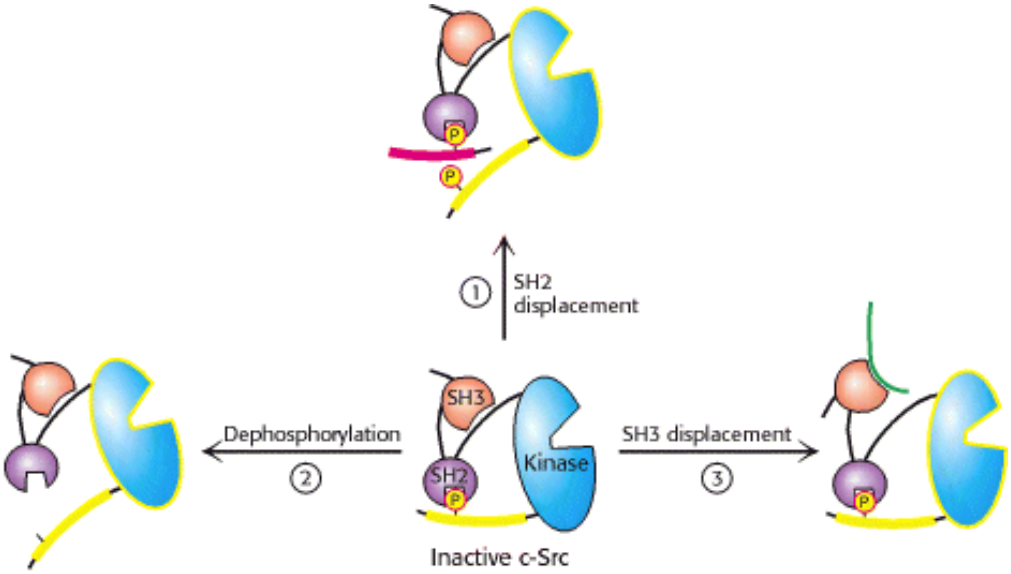


Figure 15.36. Activation Pathways for c-Src. Inactive c-Src can be activated by one of at least three distinct pathways: (1) displacement of the SH2 domain, (2) dephosphorylation, or (3) displacement of the SH3 domain.

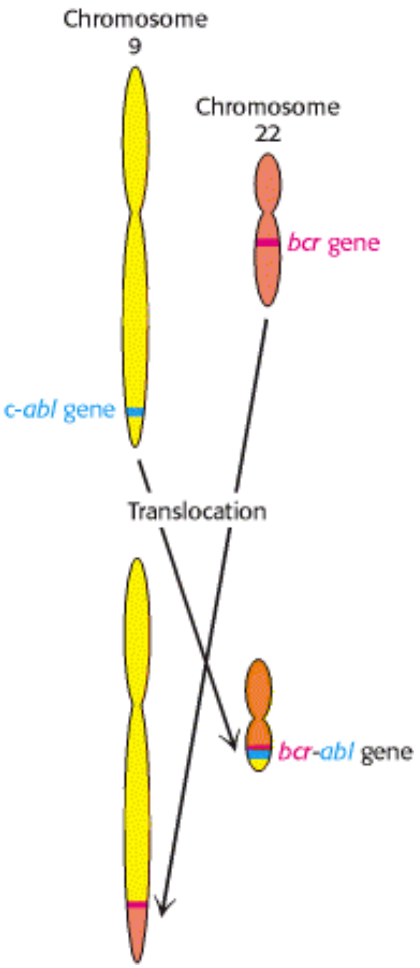


Figure 15.37. Formation of the Bcr-Abl Gene by Translocation. In chronic myologenous leukemia, parts of chromosomes 9 and 22 are reciprocally exchanged, causing the *bcr* and *abl* genes to fuse. The protein kinase encoded by the *bcr-abl* gene is expressed at higher levels in cells having this translocation than is the c-*abl* gene in normal cells.

Table 15.4. Diseases of heterotrimeric G proteins

Disease
Excessive signaling
Cholera
Cancer (adenoma) of pituitary and thyroid
Cancer (adenoma) of adrenal and ovary
Essential hypertension
Deficient signaling
Night blindness
Pseudohypoparathyroidism type Ib
Pertussis

Source: After Z. Farfel, H. R. Bourne, and T. Iiri. *N. Engl. J. Med.* 340(1999):1012.

15.6. Recurring Features of Signal-Transduction Pathways Reveal Evolutionary Relationships

Many features of signal-transduction pathways are ancient. For example, cAMP signals the need for energy in prokaryotes as well as eukaryotes, although the mechanisms for detecting cAMP are different. Similarly, the GTP-binding proteins—the G_{α} subunits of the hetero-trimeric G proteins and the members of the Ras family—are part of an ancient superfamily of evolutionarily related proteins. Other members of this superfamily are proteins that cycle between ATP- and ADP-bound forms; these proteins function in ATP synthesis (Section 18.4.5) and in generating molecular motion (Chapter 34). The superfamily also includes proteins taking part in protein synthesis (Section 29.4.2). The key feature of these proteins is that they undergo significant conformational changes on binding nucleoside triphosphates and hydrolyzing them to nucleoside diphosphates. These proteins can thus function as molecular "on-off" switches. A domain with this ability must have arisen early in evolution and been adapted to meet a range of biochemical needs since.

Other proteins crucial to signal-transduction pathways arose much later. For example, the eukaryotic protein kinases are one of the largest protein families in all eukaryotes and yet appear to be absent in prokaryotes. The evolution of the eukaryotic protein kinase domain appears to have been an important biochemical step in the appearance of eukaryotes and the subsequent development of multicellular organisms.

Entire signaling pathways have been conserved between organisms. For example, a key pathway in eye development in *Drosophila* is completely analogous to the EGF pathways in human beings (Figure 15.38). Thus, the wiring of this growth-control pathway is at least 800 million years old.

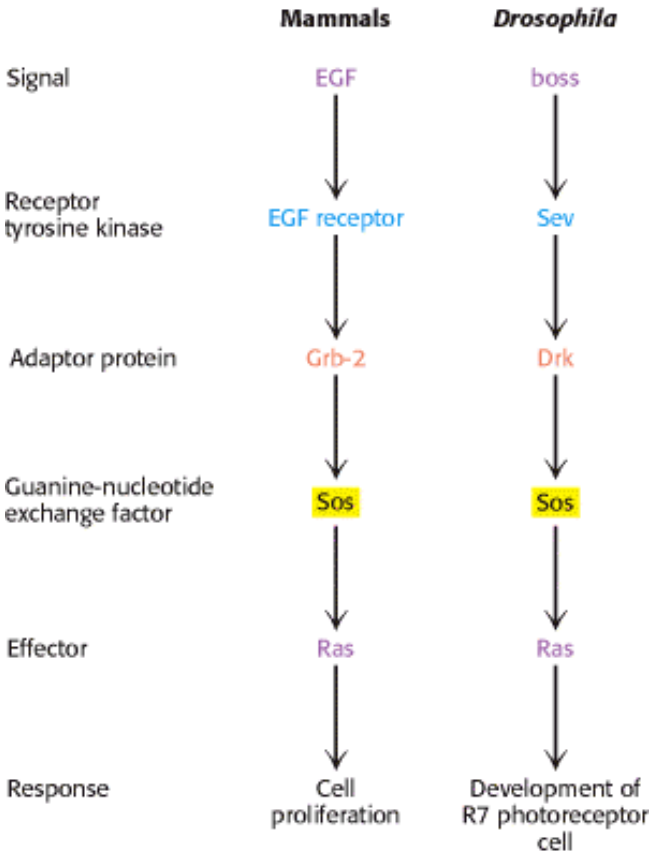


Figure 15.38. Pathway Conservation. A pathway homologous to the mammalian EGF signal-transduction pathway functions in *Drosophila* to control the development of a specific photoreceptor cell in the eye.

Summary

The highly specific binding of signal molecules, many of which are hormones and growth factors, to receptor molecules initiates the signal-transduction cascade. Secondary messengers carry the signal inside the cell and often use protein phosphorylation as a signaling device.

Seven-Transmembrane-Helix Receptors Change Conformation in Response to Ligand Binding and Activate G Proteins

Seven-transmembrane-helix receptors operate in conjunction with heterotrimeric G proteins. The binding of hormone to a 7TM receptor triggers the exchange of GTP for GDP bound to the α subunit of the G protein. G_{α} proteins can transmit information in a number of ways. $G_{\alpha s}$-GTP activates adenylate cyclase, an integral membrane protein that catalyzes the synthesis of cAMP. Cyclic AMP then activates protein kinase A by binding to its regulatory subunit, thus unleashing its catalytic chains. PKA, a multifunctional kinase, alters the activity of many target proteins by phosphorylating serine and threonine residues.

The Hydrolysis of Phosphatidyl Inositol Bisphosphate by Phospholipase C Generates Two Messengers

The phosphoinositide cascade is mediated by 7TM receptors and $G_{\alpha q}$ proteins. The receptor-triggered activation of phospholipase C generates two intracellular messengers by hydrolysis of phosphatidyl inositol 4,5-bisphosphate. Inositol trisphosphate opens calcium channels in the endoplasmic and sarcoplasmic reticulum membranes, leading to an elevated level of Ca^{2+} in the cytosol. Diacylglycerol activates protein kinase C, which phosphorylates serine and threonine residues in target proteins.

Calcium Ion Is a Ubiquitous Cytosolic Messenger

Calcium ion acts by binding to calmodulin and other calcium sensors. Calmodulin contains four calcium-binding modules called EF hands that recur in other proteins. Ca^{2+}-calmodulin activates target proteins by binding to positively charged amphipathic helices.

Some Receptors Dimerize in Response to Ligand Binding and Signal by Cross-Phosphorylation

Some ligands induce dimerization of the receptors to which they bind. Such a receptor contains an extracellular domain that binds the ligand, a transmembrane region, and a cytosolic domain that either binds or contains a protein kinase. The growth-hormone receptor participates in an example of this type of signal-transduction pathway. Dimerization of the receptor activates Janus kinase 2, a protein kinase associated with the intracellular part of the receptor. The kinase, in turn, phosphorylates and activates a transcription factor called STAT5.

Intrinsic tyrosine kinases are covalently incorporated in the intracellular domains of some receptors, such as epidermal growth factor receptor and the insulin receptor. When such receptor tyrosine kinases dimerize, cross-phosphorylation occurs. The phosphorylated tyrosines in activated receptor tyrosine kinases serve as docking sites for SH2 domains present in numerous signaling proteins and permit further propagation of the signal. A prominent component of such pathways is the small GTPase Ras. The Ras protein, like the G_{α} subunit, cycles between an inactive form bound to GDP and an active form bound to GTP.

Defects in Signaling Pathways Can Lead to Cancer and Other Diseases

If the genes encoding components of the signal-transduction pathways are altered by mutation, pathological conditions, most notably cancer, may result. In their mutated form, these genes are called oncogenes. The normal counterparts are called proto-oncogenes and function in pathways that control cell growth and replication. Mutated versions of *ras* are frequently found in human cancers.

Recurring Features of Signal-Transduction Pathways Reveal Evolutionary Relationships

Many aspects of signal transduction are ancient and appear throughout the kingdoms of life. G proteins of various classes are employed as molecular switches in a host of biochemical processes.

Key Terms

ligand

primary messenger

second messenger

protein kinase

protein phosphatase

seven-transmembrane-helix (7TM) receptor

G protein

adenylate cyclase

G_{α}

$G_{\beta\gamma}$

G-protein-coupled receptor (GPCR)

desensitization (adaptation)

protein kinase A (PKA)

phosphoinositide cascade

phospholipase C

protein kinase C (PKC)

pseudosubstrate sequence

calmodulin (CaM)

EF hand

calmodulin-dependent protein (CaM) kinase

SH2 domain

receptor tyrosine kinase (RTK)

SH3 domain

small G protein

oncogene

proto-oncogene

Problems

1. *Levels of amplification.* At which stages in the signaling pathway from epinephrine to cAMP does a significant amount of amplification occur? Answer the same question for the signaling pathways from human growth hormone to STAT5 and from EGF to Ras.

   See answer

2. *Active mutants.* Some protein kinases are inactive unless they are phosphorylated on key serine or threonine residues. In some cases, active enzymes can be generated by mutating these serine or threonine residues to glutamate. Propose an explanation.

   See answer

3. *In the pocket.* SH2 domains bind phosphotyrosine residues in deep pockets on their surfaces. Would you expect SH2 domains to bind phosphoserine or phosphothreonine with high affinity? Why or why not?

   See answer

4. *Inhibitory constraints.* Many proteins in signal-transduction pathways are activated by the removal of an inhibitory constraint. Give two examples of this recurring mechanism.

   See answer

5. *Pseudosubstrate mutant.* A mutated form of a protein kinase C isozyme that has three changes in amino acids in the pseudosubstrate sequence is isolated. What properties would you expect this mutated form to have?

   See answer

6. *Antibodies mimicking hormones.* Antibodies have two identical antigen-binding sites. Remarkably, antibodies to the extracellular parts of growth-factor receptors often lead to the same cellular effects as does exposure to growth factors. Explain this observation.

See answer

7. *Facile exchange.* A mutated form of the α subunit of the heterotrimeric G protein has been identified; this form readily exchanges nucleotides even in the absence of an activated receptor. What effect would you expect this mutated α subunit to have on its signaling pathway?

See answer

8. *Blocking one side.* Human growth hormone binds simultaneously to two growth-hormone receptors through two different surfaces. Suppose sequence changes in a mutated form of growth hormone disrupt one interaction surface but do not affect the other. What properties would such a mutated hormone have? Why might it be useful?

See answer

9. *Diffusion rates.* Normally, rates of diffusion vary inversely with molecular weights; so smaller molecules diffuse faster than do larger ones. In cells, however, calcium ion diffuses more slowly than does cAMP. Propose a possible explanation.

See answer

10. *Awash with glucose.* Glucose is mobilized for ATP generation in muscle in response to epinephrine, which activates G_{α^s}. Cyclic AMP phosphodiesterase is an enzyme that converts cAMP into AMP. How would inhibitors of cAMP phosphodiesterase affect glucose mobilization in muscle?

See answer

Chapter Integration Problem

11. *Nerve growth factor pathway.* Nerve growth factor (NGF) binds to a protein tyrosine kinase receptor. The amount of diacylglycerol in the plasma membrane increases in cells expressing this receptor when treated with NGF. Propose a simple signaling pathway and identify the isoform of any participating enzymes. Would you expect the concentrations of any other common second messengers to increase on NGF treatment?

See answer

Mechanism Problem

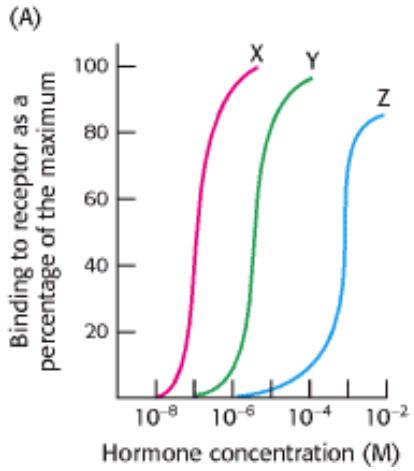
12. *Distant relatives.* The structure of adenylate cyclase is similar to the structures of some types of DNA polymerases, suggesting that these enzymes derived from a common ancestor. Compare the reactions catalyzed by these two enzymes. In what ways are they similar?

See answer

Data Interpretation Problems

13. *Establishing specificity.* You wish to determine the hormone-binding specificity of a newly identified membrane receptor. Three different hormones, X, Y, and Z, were mixed with the receptor in separate experiments, and the percentage of binding capacity of the receptor was determined as a function of hormone concentration, as shown in graph A.

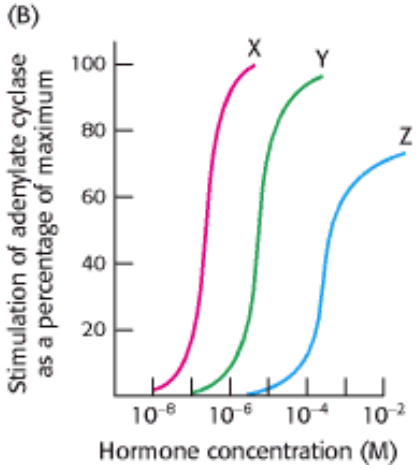
(a) What concentrations of each hormone yield 50% maximal binding?



(b) Which hormone shows the highest binding affinity for the receptor?

You next wish to determine whether the hormone-receptor complex stimulates the adenylate cyclase cascade. To do so, you measure adenylate cyclase activity as a function of hormone concentration, as shown in graph B.

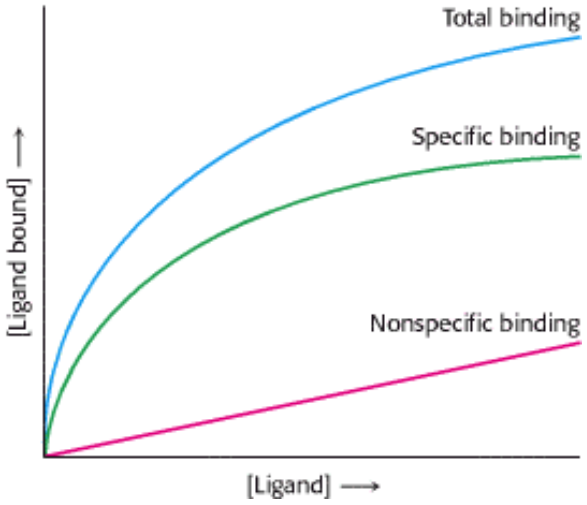
(c) What is the relation between the binding affinity of the hormone-receptor complex and the ability of the hormone to enhance adenylate cyclase activity? What can you conclude about the mechanism of action of the hormone-receptor complex?



(d) Suggest experiments that would determine whether a $G_{\alpha s}$ protein is a component of the signal-transduction pathway.

See answer

14. *Binding issues.* A scientist wishes to determine the number of receptors specific for a ligand X, which he has in both radioactive and nonradioactive form. In one experiment, he adds increasing amounts of the radioactive X and measures how much of it is bound to the cells. The result is shown as total activity in the adjoining graph. Next, he performs the same experiment, except that he includes a several hundredfold excess of nonradioactive X. This result is shown as nonspecific binding. The difference between the two curves is the specific binding.



(a) Why is the total binding not an accurate representation of the number of receptors on the cell surface?

(b) What is the purpose of performing the experiment in the presence of excess nonradioactive ligand?

(c) What is the significance of the fact that specific binding attains a plateau?

See answer

15. *Counting receptors.* With the use of experiments such as those described in problems 13 and 14, it is possible to calculate the number of receptors in the cell membrane. Suppose that the specific activity of the ligand is 10^{12} cpm per millimole and that the maximal specific binding is 10^4 cpm per milligram of membrane protein. There are 10^{10} cells per milligram of membrane protein. Assume that one ligand binds per receptor. Calculate the number of receptor molecules present per cell.

See answer

Media Problem

16. *Grubby binding.* The **Structural Insights** module on SH2 domains describes some of the determinants of SH2 specificity and the ways in which SH2-phosphotyrosine binding can affect protein function. Given that the Src kinase SH2 domain binds Src phosphotyrosine 527, what effect do you think mutation of Glu 529 to Asn would have on the protein kinase activity of Src? Suppose you now obtained a second mutation within Src that reversed the effect of the first. Can you predict what that second mutation might be?

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16. Glycolysis and Gluconeogenesis

The first metabolic pathway that we encounter is *glycolysis*, an ancient pathway employed by a host of organisms. *Glycolysis is the sequence of reactions that metabolizes one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP*. This process is anaerobic (i.e., it does not require O_2) inasmuch as it evolved before the accumulation of substantial amounts of oxygen in the atmosphere. Pyruvate can be further processed anaerobically (fermented) to lactate (*lactic acid fermentation*) or ethanol (*alcoholic fermentation*). Under aerobic conditions, pyruvate can be completely oxidized to CO_2, generating much more ATP, as will be discussed in Chapters 17 and 18.

Glucose can be synthesized from noncarbohydrate precursors, such as pyruvate and lactic acid, in the process of *gluconeogenesis*. Although glycolysis and gluconeogenesis have some of the same enzymes in common, the two pathways are not simply the reverse of each other. In particular, the highly exergonic, irreversible steps of glycolysis are bypassed in gluconeogenesis. Both pathways are stringently controlled by intercellular and intracellular signals, and they are reciprocally regulated so that glycolysis and gluconeogenesis do not take place simultaneously in the same cell to a significant extent.

Our understanding of glucose metabolism, especially glycolysis, has a rich history. Indeed, the development of biochemistry and the delineation of glycolysis went hand in hand. A key discovery was made by Hans Buchner and Eduard Buchner in 1897, quite by accident. The Buchners were interested in manufacturing cell-free extracts of yeast for possible therapeutic use. These extracts had to be preserved without the use of antiseptics such as phenol, and so they decided to try sucrose, a commonly used preservative in kitchen chemistry. They obtained a startling result: sucrose was rapidly fermented into alcohol by the yeast juice. The significance of this finding was immense. *The Buchners demonstrated for the first time that fermentation could take place outside living cells*. The accepted view of their day, asserted by Louis Pasteur in 1860, was that fermentation is inextricably tied to living cells. The chance discovery of the Buchners refuted this vitalistic dogma and opened the door to modern biochemistry. *Metabolism became chemistry*.

Glycolysis

Derived from the Greek stem *glyk-*, "sweet," and the word *lysis*, "dissolution."

Studies of muscle extracts then showed that many of the reactions of lactic acid fermentation were the same as those of alcoholic fermentation. *This exciting discovery revealed an underlying unity in biochemistry*. The complete glycolytic pathway was elucidated by 1940, largely through the pioneering contributions of Gustav Embden, Otto Meyerhof, Carl Neuberg, Jacob Parnas, Otto Warburg, Gerty Cori, and Carl Cori. Glycolysis is also known as the *Embden-Meyerhof pathway*.

Enzyme

A term coined by Friedrich Whilhelm Kühne in 1878 to designate catalytically active substances that had previously been called ferments. Derived from the Greek workds *en*, "in," and *zyme*, "leaven."

In our consideration of the glycolytic and gluconeogenic pathways, we shall examine the mechanisms of selected enzymes in some detail. Of particular interest will be the enzymes that play the most central roles in converting one type

of chemical energy into another.

16.0.1. Glucose Is an Important Fuel for Most Organisms

Glucose is an important and common fuel. In mammals, glucose is the only fuel that the brain uses under nonstarvation conditions and the only fuel that red blood cells can use at all. Indeed, almost all organisms use glucose, and most that do process it in a similar fashion. Recall from Chapter 11 that there are many carbohydrates. Why is glucose instead of some other monosaccharide such a prominent fuel? We can speculate on the reasons. First, glucose is one of the monosaccharides formed from formaldehyde under prebiotic conditions, so it may have been available as a fuel source for primitive biochemical systems. Second, glucose has a low tendency, relative to other monosaccharides, to nonenzymatically glycosylate proteins. In their open-chain (carbonyl) forms, monosaccharides can react with the amino groups of proteins to form Schiff bases, which rearrange to form a more stable amino ketone linkage. Such nonspecifically modified proteins often do not function effectively. Glucose has a strong tendency to exist in the ring formation and, consequently, relatively little tendency to modify proteins. Recall that all the hydroxyl groups in the ring conformation of β-glucose are equatorial, contributing to this high relative stability (Section 11.12.12).

16.0.2. Fermentations Provide Usable Energy in the Absence of Oxygen

Although glycolysis is a nearly universal process, the fate of its end product, pyruvate, may vary in different organisms or even in different tissues. In the presence of oxygen, the most common situation in multicellular organisms and many unicellular ones, pyruvate is metabolized to carbon dioxide and water through the citric acid cycle and the electron-transport chain. In the absence of oxygen, fermentation generates a lesser amount of energy; pyruvate is converted, or fermented, into lactic acid in lactic acid fermentation or into ethanol in alcoholic fermentation (Figure 16.1). Lactic acid production takes place in skeletal muscle when energy needs outpace the ability to transport oxygen. Although we will consider only these two fermentations, microorganisms are capable of generating a wide array of molecules as end points to fermentation (Table 16.1). Indeed, many food products are the result of fermentations. These foods include sour cream, yogurt, various cheeses, beer, wine, and sauerkraut.

Fermentation

An ATP-generating process in which organic compounds act as both donors and acceptors of electrons. Fermentation can take place in the absence of O_2. Discovered by Louis Pasteur, who described fermentation as "la vie sans l'air" ("life without air").

Fermentations yield only a fraction of the energy available from the complete combustion of glucose. Why is a relatively inefficient metabolic pathway so extensively used? The fundamental reason is that oxygen is not required. The ability to survive without oxygen affords a host of living accommodations such as soils, deep water, and skin pores. Some organisms, called *obligate anaerobes*, cannot survive in the presence of O_2, a highly reactive compound. The bacterium *Clostridium perfringens*, the cause of gangrene, is an example of an obligate anaerobe. Other pathogenic obligate anaerobes are listed in Table 16.2.

Facultative anaerobes can function in the presence or absence of oxygen. For instance, organisms that live in the intertidal zone, such as the bivalve *Mytilus* (Figure 16.2), can function aerobically, using gills when they are under water and anaerobically when exposed to the air. Such organisms display habitat-dependent anaerobic functioning, or *habitat-dependent anaerobiosis*. Muscles in most animals display *activity-dependent anaerobiosis*, meaning that they can function anaerobically for short periods. For example, when animals perform bursts of intense exercise, their ATP needs rise faster than the ability of the body to provide oxygen to the muscle. The muscle functions anaerobically until the lactic acid builds up to the point at which the fall in pH inhibits the anaerobic pathway (Section 16.2.1).

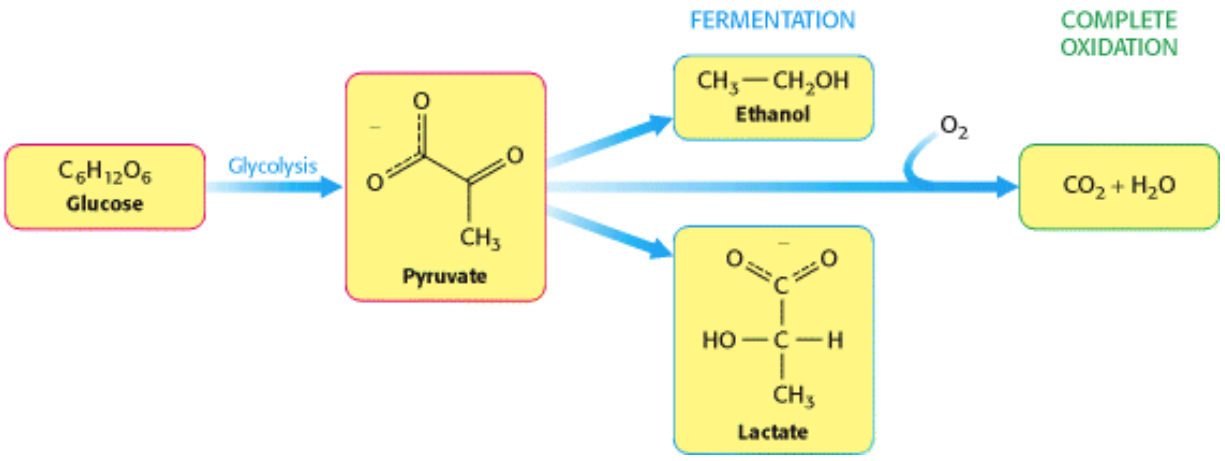


Figure 16.1. Some Fates of Glucose.

Table 16.1. Starting and ending points of various fermentations

TABLE 16.1 Starting and ending points of various fermentations

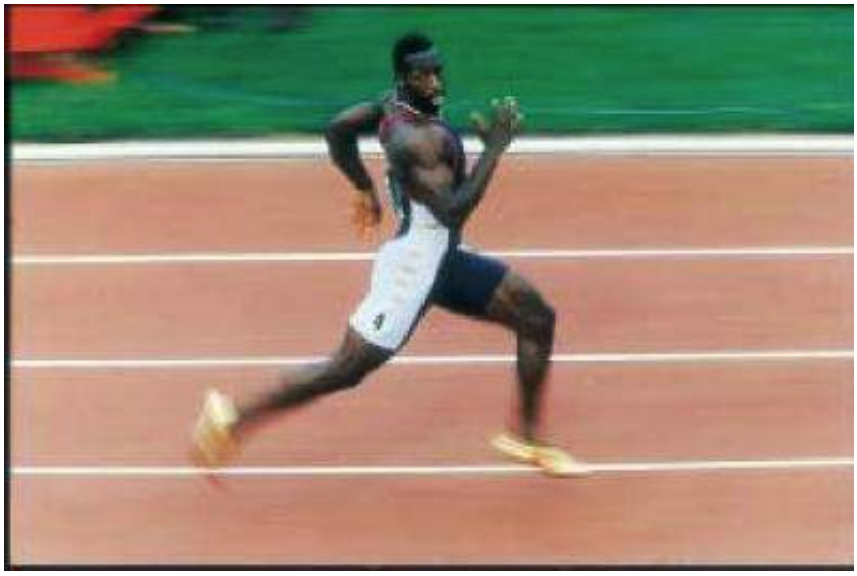
Glucose	→	lactate
Lactate	→	acetate
Glucose	→	ethanol
Ethanol	→	acetate
Arginine	→	carbon dioxide
Pyrimidines	→	carbon dioxide
Purines	→	formate
Ethylene glycol	→	acetate
Threonine	→	propionate
Leucine	→	2-alkylacetate
Phenylalanine	→	propionate

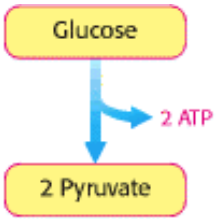
Note: The products of some fermentations are the substrates for others.

Table 16.2. Examples of pathogenic obligate anaerobes

Bacterium	Results of infection
Clostridium tetani	Tetanus (lockjaw)
Clostridium botulinum	Botulism (an especially severe type of food poisoning)
Clostridium perfringens	Gas gangrene (gas is produced as an end point of the fermentation, distorting and destroying the tissue)
Bartonella hensela	Cat scratch fever (flulike symptoms)
Bacteroides fragilis	Abdominal, pelvic, pulmonary, and blood infections

Figure 16.2. The Bivalve *Mytilus*. These mussels, inhabitants of the intertidal zone, display habitat-dependent anaerobiosis. [Ed Reschke/Peter Arnold.]





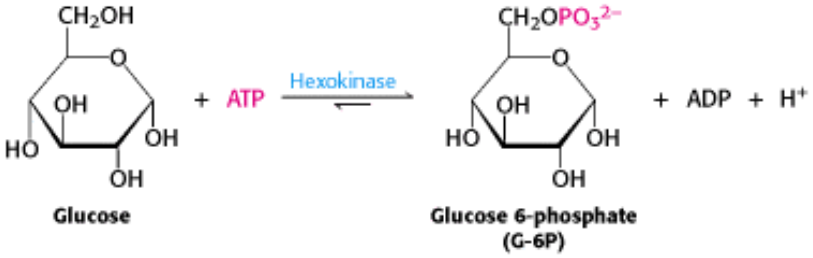
Glycolysis produces energy. Michael Johnson sprints to another victory in the 200-meter semifinals of the Olympics. Johnson, like anyone who sprints, requires a source of energy that can be rapidly accessed. The anaerobic metabolism of glucose—the process of glycolysis—provides such a source of energy for short, intense bouts of exercise. [Simon Bruty/ Allsport.]

16.1. Glycolysis Is an Energy-Conversion Pathway in Many Organisms

We now start our consideration of the glycolytic pathway. This pathway is common to virtually all cells, both prokaryotic and eukaryotic. In eukaryotic cells, glycolysis takes place in the cytosol. This pathway can be thought of as comprising three stages (Figure 16.3). Stage 1, which is the conversion of glucose into fructose 1,6-bisphosphate, consists of three steps: a phosphorylation, an isomerization, and a second phosphorylation reaction. *The strategy of these initial steps in glycolysis is to trap the glucose in the cell and form a compound that can be readily cleaved into phosphorylated three-carbon units*. Stage 2 is the cleavage of the fructose 1,6-bisphosphate into two three-carbon fragments. These resulting three-carbon units are readily interconvertible. In stage 3, ATP is harvested when the three-carbon fragments are oxidized to pyruvate.

16.1.1. Hexokinase Traps Glucose in the Cell and Begins Glycolysis

Glucose enters cells through specific transport proteins (Section 16.2.4) and has one principal fate: *it is phosphorylated by ATP to form glucose 6-phosphate*. This step is notable for two reasons: (1) glucose 6-phosphate cannot diffuse through the membrane, because of its negative charges, and (2) the addition of the phosphoryl group begins to destabilize glucose, thus facilitating its further metabolism. The transfer of the phosphoryl group from ATP to the hydroxyl group on carbon 6 of glucose is catalyzed by *hexokinase*.



Phosphoryl transfer is a fundamental reaction in biochemistry and is one that was discussed in mechanistic and structural detail earlier (Section 9.4). *Kinases* are enzymes that catalyze the transfer of a phosphoryl group from ATP to an acceptor. Hexokinase, then, catalyzes the transfer of a phosphoryl group from ATP to a variety of six-carbon sugars (*hexoses*), such as glucose and mannose. *Hexokinase, like adenylate kinase* (Section 9.4.2) *and all other kinases, requires* Mg^{2+} *(or another divalent metal ion such as* Mn^{2+}*) for activity*. The divalent metal ion forms a complex with ATP.

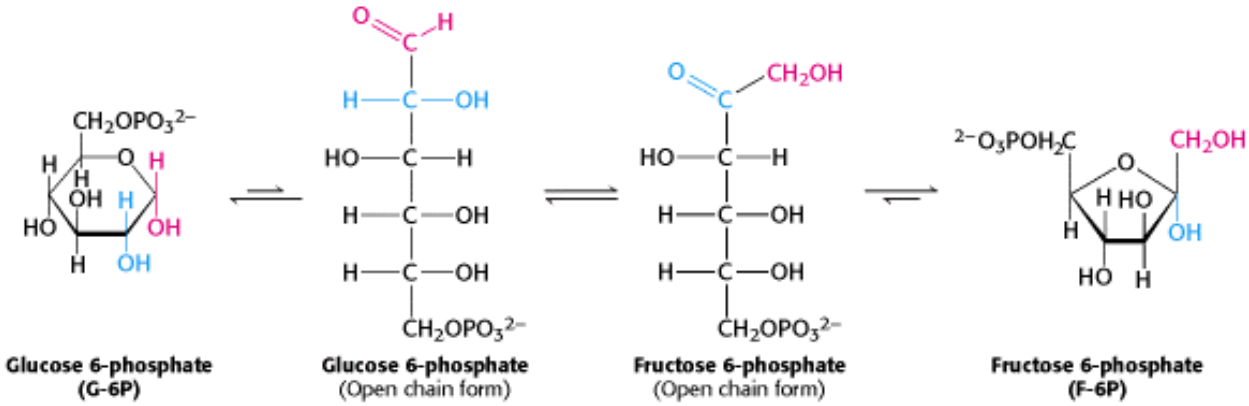
The results of x-ray crystallographic studies of yeast hexokinase revealed that the binding of glucose induces a large conformational change in the enzyme, analogous to the conformational changes undergone by NMP kinases on substrate binding (Section 9.4.3). Hexokinase consists of two lobes, which move toward each other when glucose is bound (Figure 16.4). On glucose binding, one lobe rotates 12 degrees with respect to the other, resulting in movements of the polypeptide backbone of as much as 8 Å. The cleft between the lobes closes, and the bound glucose becomes surrounded by protein, except for the hydroxyl group of carbon 6, which will accept the phosphoryl group from ATP. The closing of the cleft in hexokinase is a striking example of the role of *induced fit* in enzyme action (Section 8.3.2).

The glucose-induced structural changes are significant in two respects. First, the environment around the glucose becomes much more nonpolar, which favors the donation of the terminal phosphoryl group of ATP. Second, as noted in Section 9.4.3, the substrate-induced conformational changes within the kinase enables it to discriminate against H_2O as a substrate. If hexokinase were rigid, a molecule of H_2O occupying the binding site for the-CH_2OH of glucose would attack the γ phosphoryl group of ATP, forming ADP and P_i. In other words, a rigid kinase would necessarily also be an ATPase. It is interesting to note that other kinases taking part in glycolysis—pyruvate kinase, phosphoglycerate kinase, and phosphofructokinase—also contain clefts between lobes that close when substrate is bound, although the structures

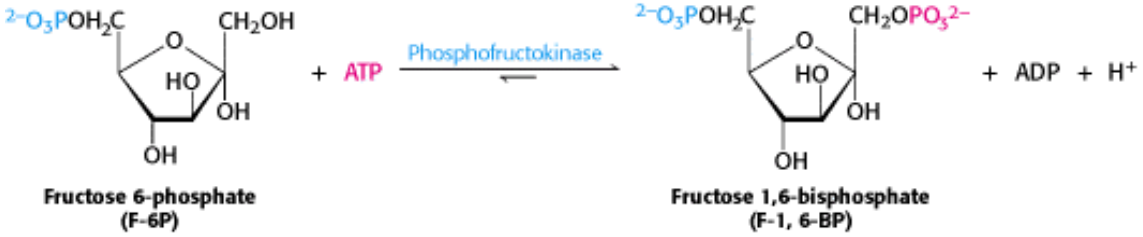
of these enzymes are different in other regards. *Substrate-induced cleft closing is a general feature of kinases*.

16.1.2. The Formation of Fructose 1,6-bisphosphate from Glucose 6-phosphate

The next step in glycolysis is the *isomerization of glucose 6-phosphate to fructose 6-phosphate*. Recall that the open-chain form of glucose has an aldehyde group at carbon 1, whereas the open-chain form of fructose has a keto group at carbon 2. Thus, the isomerization of glucose 6-phosphate to fructose 6-phosphate is a *conversion of an aldose into a ketose*. The reaction catalyzed by *phosphoglucose isomerase* includes additional steps because both glucose 6-phosphate and fructose 6-phosphate are present primarily in the cyclic forms. The enzyme must first open the six-membered ring of glucose 6-phosphate, catalyze the isomerization, and then promote the formation of the five-membered ring of fructose 6-phosphate.



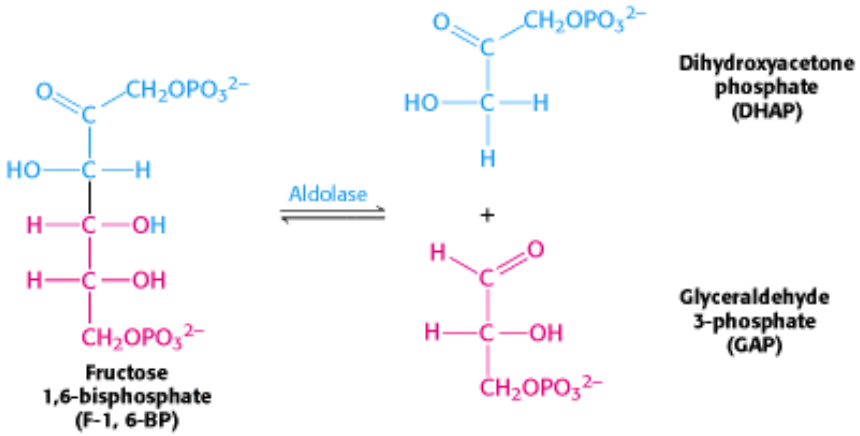
A second phosphorylation reaction follows the isomerization step. *Fructose 6-phosphate is phosphorylated by ATP to fructose 1,6-bisphosphate* (F-1,6-BP). The prefix *bis-* in bisphosphate means that two separate monophosphate groups are present, whereas the prefix *di-* in diphosphate (as in adenosine diphosphate) means that two phosphate groups are present and are connected by an anhydride bond.



This reaction is catalyzed by *phosphofructokinase* (PFK), an allosteric enzyme that sets the pace of glycolysis (Section 16.2.1). As we will learn, this enzyme plays a central role in the integration of much of metabolism.

16.1.3. The Six-Carbon Sugar Is Cleaved into Two Three-Carbon Fragments by Aldolase

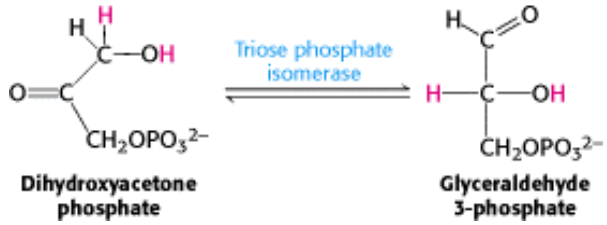
The second stage of glycolysis begins with the splitting of fructose 1,6-bisphosphate into *glyceraldehyde 3-phosphate* (*GAP*) and *dihydroxyacetone phosphate* (*DHAP*). The products of the remaining steps in glycolysis consist of three-carbon units rather than six-carbon units.



This reaction is catalyzed by *aldolase*. This enzyme derives its name from the nature of the reverse reaction, an aldol condensation. The reaction catalyzed by aldolase is readily reversible under intracellular conditions.

16.1.4. Triose phosphate isomerase Salvages a Three-Carbon Fragment

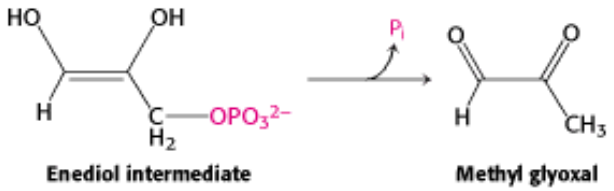
Glyceraldehyde 3-phosphate is on the direct pathway of glycolysis, whereas dihydroxyacetone phosphate is not. Unless a means exists to convert dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, a three-carbon fragment useful for generating ATP will be lost. These compounds are isomers that can be readily interconverted: dihydroxyacetone phosphate is a ketose, whereas glyceraldehyde 3-phosphate is an aldose. The isomerization of these three-carbon phosphorylated sugars is catalyzed by *triose phosphate isomerase* (*TIM*; Figure 16.5). This reaction is rapid and reversible. At equilibrium, 96% of the triose phosphate is dihydroxyacetone phosphate. However, the reaction proceeds readily from dihydroxyacetone phosphate to glyceraldehyde 3-phosphate because the subsequent reactions of glycolysis remove this product.



Much is known about the catalytic mechanism of triose phosphate isomerase. TIM catalyzes the transfer of a hydrogen atom from carbon 1 to carbon 2 in converting dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, an intramolecular oxidation-reduction. This isomerization of a ketose into an aldose proceeds through an *enediol intermediate* (Figure 16.6).

X-ray crystallographic and other studies showed that glutamate 165 (see Figure 16.5) plays the role of a general acid-base catalyst. However, this carboxylate group by itself is not basic enough to pull a proton away from a carbon atom adjacent to a carbonyl group. Histidine 95 assists catalysis by donating a proton to stabilize the negative charge that develops on the C-2 carbonyl group.

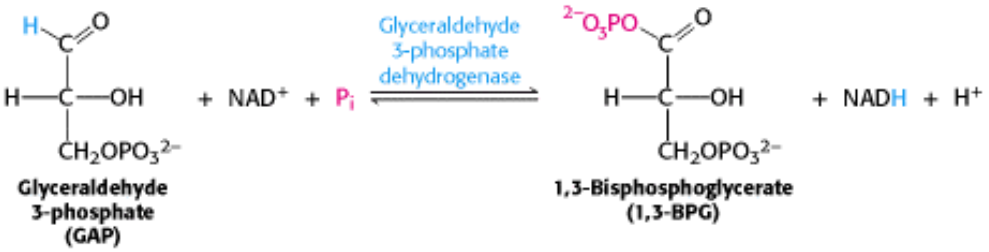
Two features of this enzyme are noteworthy. First, TIM displays great catalytic prowess. It accelerates isomerization by a factor of 10^{10} compared with the rate obtained with a simple base catalyst such as acetate ion. Indeed, the k_{cat}/K_M ratio for isomerization of glyceraldehyde 3-phosphate is $2 \times 10^8\ M^{-1}\ s^{-1}$, which is close to the diffusion-controlled limit. In other words, the rate-limiting step in catalysis is the diffusion-controlled encounter of substrate and enzyme. TIM is an example of a *kinetically perfect enzyme* (Section 8.2.5). Second, TIM suppresses an undesired side reaction, the decomposition of the enediol intermediate into methyl glyoxal and inorganic phosphate.



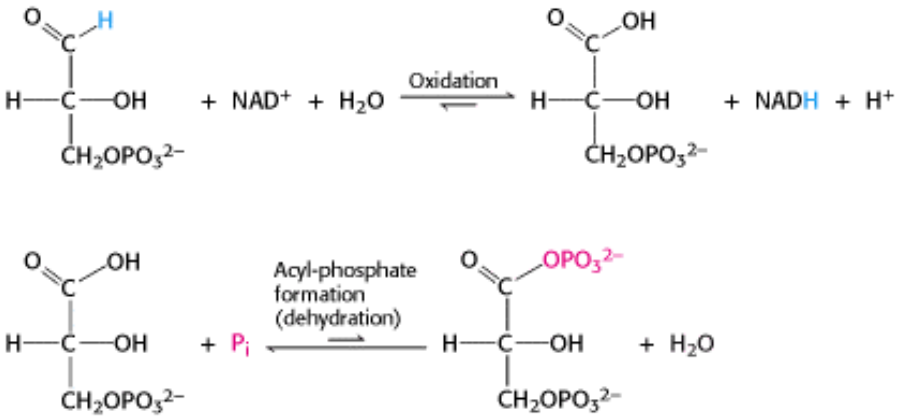
In solution, this physiologically useless reaction is 100 times as fast as isomerization. Hence, TIM must prevent the enediol from leaving the enzyme. This labile intermediate is trapped in the active site by the movement of a loop of 10 residues (see Figure 16.5). This loop serves as a lid on the active site, shutting it when the enediol is present and reopening it when isomerization is completed. *We see here a striking example not only of catalytic perfection, but also of the acceleration of a desirable reaction so that it takes place much faster than an undesirable alternative reaction*. Thus, two molecules of glyceraldehyde 3-phosphate are formed from one molecule of fructose 1,6-bisphosphate by the sequential action of aldolase and triose phosphate isomerase. The economy of metabolism is evident in this reaction sequence. The isomerase funnels dihydroxyacetone phosphate into the main glycolytic pathway—a separate set of reactions is not needed.

16.1.5. Energy Transformation: Phosphorylation Is Coupled to the Oxidation of Glyceraldehyde 3-phosphate by a Thioester Intermediate

The preceding steps in glycolysis have transformed one molecule of glucose into two molecules of glyceraldehyde 3-phosphate, but no energy has yet been extracted. On the contrary, thus far two molecules of ATP have been invested. We come now to a series of steps that harvest some of the energy contained in glyceraldehyde 3-phosphate. The initial reaction in this sequence is the *conversion of glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate* (*1,3-BPG*), a reaction catalyzed by *glyceraldehyde 3-phosphate dehydrogenase* (Figure 16.7).



1,3-Bisphosphoglycerate is an acyl phosphate. Such compounds have a high phosphoryl-transfer potential; one of its phosphoryl groups is transferred to ADP in the next step in glycolysis. The reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase is really the sum of two processes: the *oxidation* of the aldehyde to a carboxylic acid by NAD^+ and the *joining* of the carboxylic acid and orthophosphate to form the acyl-phosphate product.



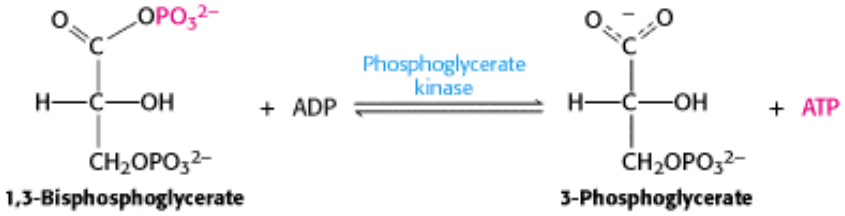
The first reaction is quite thermodynamically favorable with a standard free-energy change, $\Delta G^{\circ\prime}$, of approximately -12 kcal mol^{-1} (-50 kJ mol^{-1}), whereas the second reaction is quite unfavorable with a standard free-energy change of the same magnitude but the opposite sign. If these two reactions simply took place in succession, the second reaction would have a very large activation energy and thus not take place at a biologically significant rate. These two processes *must be coupled* so that the favorable aldehyde oxidation can be used to drive the formation of the acyl phosphate. How are these reactions coupled? The key is an intermediate, formed as a result of the aldehyde oxidation, that is higher in free energy than the free carboxylic acid is. This intermediate reacts with orthophosphate to form the acyl-phosphate product.

Let us consider the mechanism of glyceraldehyde 3-phosphate dehydrogenase in detail (Figure 16.8). In step 1, the aldehyde substrate reacts with the sulfhydryl group of cysteine 149 on the enzyme to form a hemithioacetal. Step 2 is the *transfer of a hydride ion to a molecule of NAD $^+$ that is tightly bound to the enzyme and is adjacent to the cysteine residue*. This reaction is favored by the deprotonation of the hemithioacetal by histidine 176. The products of this reaction are the reduced coenzyme NADH and a thioester intermediate. *This thioester intermediate has a free energy close to that of the reactants*. In step 3, orthophosphate attacks the thioester to form 1,3-BPG and free the cysteine residue. This displacement occurs only after the NADH formed from the aldehyde oxidation has left the enzyme and been replaced by a second NAD^+. The positive charge on the NAD^+ may help polarize the thioester intermediate to facilitate the attack by orthophosphate.

This example illustrates the essence of energy transformations and of metabolism itself: energy released by carbon oxidation is converted into high phosphoryl-transfer potential. The favorable oxidation and unfavorable phosphorylation reactions are *coupled by the thioester intermediate*, which preserves much of the free energy released in the oxidation reaction. We see here the *use of a covalent enzyme-bound intermediate as a mechanism of energy coupling*. A free-energy profile of the glyceraldehyde 3-phosphate dehydrogenase reaction, compared with a hypothetical process in which the reaction proceeds without this intermediate, reveals how this intermediate allows a favorable process to drive an unfavorable one (Figure 16.9).

16.1.6. The Formation of ATP from 1,3-Bisphosphoglycerate

The final stage in glycolysis is the generation of ATP from the phosphorylated three-carbon metabolites of glucose. *Phosphoglycerate kinase* catalyzes the transfer of the phosphoryl group from the acyl phosphate of 1,3-bisphosphoglycerate to ADP. ATP and 3-phosphoglycerate are the products.



The formation of ATP in this manner is referred to as *substrate-level phosphorylation* because the phosphate donor, 1,3-BPG, is a substrate with high phosphoryl-transfer potential. We will contrast this manner of ATP formation with that in which ATP is formed from ionic gradients in Chapters 18 and 19.

Thus, the outcomes of the reactions catalyzed by glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase are:

1. Glyceraldehyde 3-phosphate, an aldehyde, is oxidized to 3-phosphoglycerate, a carboxylic acid.

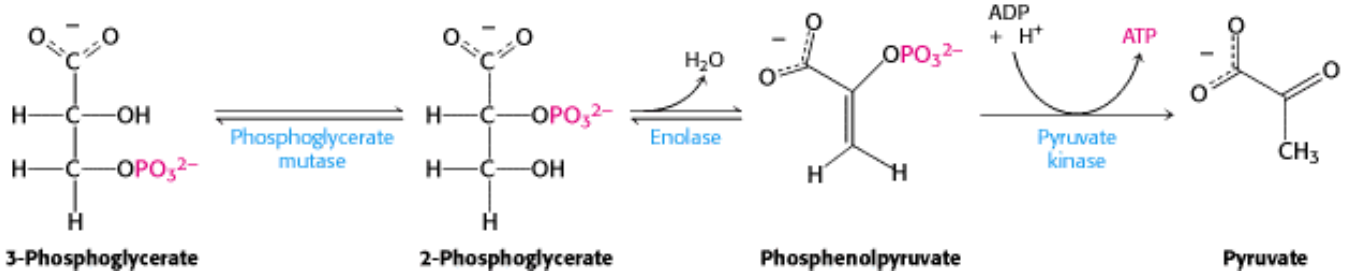
2. NAD^+ is concomitantly reduced to NADH.

3. ATP is formed from P_i and ADP at the expense of carbon oxidation energy.

Keep in mind that, because of the actions of aldolase and triose phosphate isomerase, two molecules of glyceraldehyde 3-phosphate were formed and hence two molecules of ATP were generated. These ATP molecules make up for the two molecules of ATP consumed in the first stage of glycolysis.

16.1.7. The Generation of Additional ATP and the Formation of Pyruvate

In the remaining steps of glycolysis, 3-phosphoglycerate is converted into pyruvate with the concomitant conversion of ADP into ATP.



The first reaction is a rearrangement. The position of the phosphoryl group shifts in the conversion of *3-phosphoglycerate into 2-phosphoglycerate,* a reaction catalyzed by *phosphoglycerate mutase*. In general, a *mutase* is an enzyme that catalyzes the intramolecular shift of a chemical group, such as a phosphoryl group. The phosphoglycerate mutase reaction has an interesting mechanism: the phosphoryl group is not simply moved from one carbon to another. This enzyme requires catalytic amounts of 2,3-bisphosphoglycerate to maintain an active-site histidine residue in a phosphorylated form.

$$\text{Enz-His} + \text{2,3 bisphosphoglycerate} \rightleftharpoons \text{Enz-His-phosphate} + \text{2-phosphoglycerate}$$

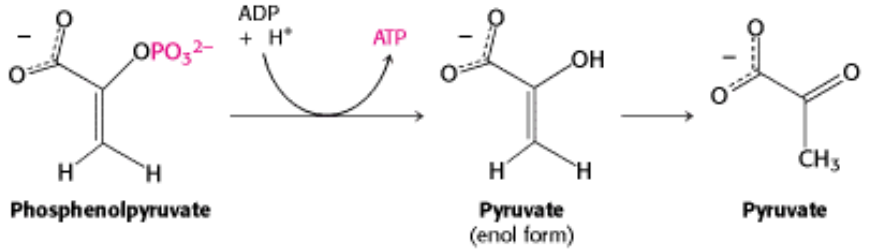
$$\text{Enz-His-phosphate} + \text{3-phosphoglycerate} \rightleftharpoons \text{Enz-His} + \text{2,3-bisphosphoglycerate}$$

The sum of these reactions yields the mutase reaction:

$$\text{3-Phosphoglycerate} \rightleftharpoons \text{2-phosphoglycerate}$$

Examination of the first partial reaction reveals that the mutase functions as a phosphatase—it converts 2,3-bisphosphoglycerate into 2-phosphoglycerate. However, the phosphoryl group remains linked to the enzyme. This phosphoryl group is then transferred to 3-phosphoglycerate to reform 2,3-bisphosphoglycerate.

In the next reaction, an *enol* is formed by the dehydration of 2-phosphoglycerate. *Enolase* catalyzes the formation of *phosphoenolpyruvate* (PEP). This dehydration markedly elevates the transfer potential of the phosphoryl group. An *enol phosphate* has a high phosphoryl-transfer potential, whereas the phosphate ester, such as 2-phosphoglycerate, of an ordinary alcohol has a low one. The $\Delta G^{\circ\prime}$ of the hydrolysis of a phosphate ester of an ordinary alcohol is -3 kcal mol^{-1} (-13 kJ mol^{-1}), whereas that of phosphoenolpyruvate is -14.8 kcal mol^{-1} (- 62 kJ mol^{-1}). Why does phosphoenolpyruvate have such a high phosphoryl-transfer potential? The phosphoryl group traps the molecule in its unstable enol form. When the phosphoryl group has been donated to ATP, the enol undergoes a conversion into the more stable ketone—namely, pyruvate.



Thus, *the high phosphoryl-transfer potential of phosphoenolpyruvate arises primarily from the large driving force of the subsequent enol-ketone conversion*. Hence, pyruvate is formed, and ATP is generated concomitantly. The virtually irreversible transfer of a phosphoryl group from phosphoenolpyruvate to ADP is catalyzed by *pyruvate kinase*. Because the molecules of ATP used in forming fructose 1,6-bisphosphate have already been regenerated, the two molecules of ATP generated from phosphoenolpyruvate are "profit."

16.1.8. Energy Yield in the Conversion of Glucose into Pyruvate

The net reaction in the transformation of glucose into pyruvate is:

$$\text{Glucose} + 2\ P_i + 2\ \text{ADP} + 2\ \text{NAD}^+ \longrightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}^+ + 2\ \text{H}_2\text{O}$$

Thus, *two molecules of ATP are generated in the conversion of glucose into two molecules of pyruvate*. The reactions of glycolysis are summarized in Table 16.3.

Note that the energy released in the anaerobic conversion of glucose into two molecules of pyruvate is -21 kcal mol^{-1} (-88 kJ mol^{-1}). We shall see in Chapters 17 and 18 how much more energy can be released from glucose in the presence of oxygen.

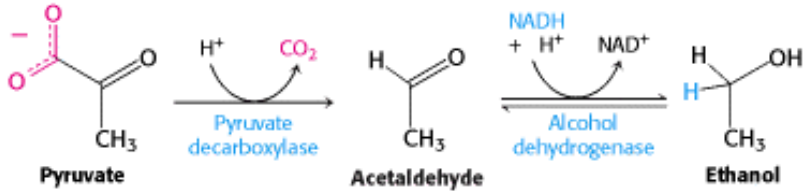
Conceptual Insights, Energetics of Glucose Metabolism. See the section on the energetics of glycolysis in the conceptual insights module for a graphical representation of free energy differences among glycolytic metabolites, and how these differences are used to drive ATP and NADH synthesis in coupled reactions.

16.1.9. Maintaining Redox Balance: The Diverse Fates of Pyruvate

The conversion of glucose into two molecules of pyruvate has resulted in the net synthesis of ATP. However, an energy-converting pathway that stopped at pyruvate would not proceed for long, because redox balance has not been maintained. As we have seen, the activity of glyceraldehyde 3-phosphate dehydrogenase, in addition to generating a compound with high phosphoryl-transfer potential, of necessity leads to the reduction of NAD^+ to NADH. There are limited amounts of NAD^+ in the cell, which is derived from the vitamin *niacin*, a dietary requirement in human beings. Consequently, NAD^+ must be regenerated for glycolysis to proceed. Thus, the final process in the pathway is the regeneration of NAD^+ through the metabolism of pyruvate. The sequence of reactions from glucose to pyruvate is similar in most organisms and most types of cells. In contrast, the fate of pyruvate is variable. Three reactions of pyruvate are of prime importance: conversion into ethanol, lactic acid, or carbon dioxide (Figure 16.10).

1. *Ethanol* is formed from pyruvate in yeast and several other microorganisms. The first step is the decarboxylation of pyruvate. This reaction is catalyzed by *pyruvate decarboxylase*, which requires the coenzyme thiamine pyrophosphate. This coenzyme, derived from the vitamin thiamine (B_1), also participates in reactions catalyzed by other enzymes (Section 17.1.1). The second step is the reduction of acetaldehyde to ethanol by NADH, in a reaction catalyzed by

alcohol dehydrogenase. This process regenerates NAD^+.



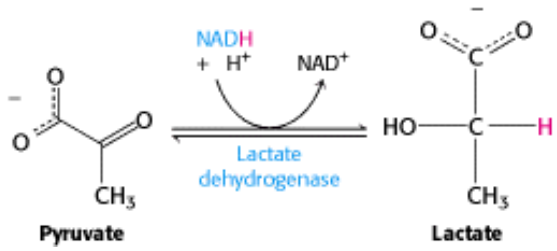
The active site of alcohol dehydrogenase contains a zinc ion that is coordinated to the sulfur atoms of two cysteine residues and a nitrogen atom of histidine (Figure 16.11). This zinc ion polarizes the carbonyl group of the substrate to favor the transfer of a hydride from NADH.

The conversion of glucose into ethanol is an example of *alcoholic fermentation*. The net result of this anaerobic process is:

$$\text{Glucose} + 2\ P_i + 2\ \text{ADP} + 2\ H^+ \longrightarrow 2\ \text{ethanol} + 2\ CO_2 + 2\ \text{ATP} + 2\ H_2O$$

Note that NAD^+ and NADH do not appear in this equation, even though they are crucial for the overall process. NADH generated by the oxidation of glyceraldehyde 3-phosphate is consumed in the reduction of acetaldehyde to ethanol. Thus, *there is no net oxidation-reduction in the conversion of glucose into ethanol* (Figure 16.12). The ethanol formed in alcoholic fermentation provides a key ingredient for brewing and winemaking.

2. *Lactate* is formed from pyruvate in a variety of microorganisms in a process called *lactic acid fermentation*. The reaction also takes place in the cells of higher organisms when the amount of oxygen is limiting, as in muscle during intense activity. The reduction of pyruvate by NADH to form lactate is catalyzed by *lactate dehydrogenase*.



The overall reaction in the conversion of glucose into lactate is:

$$\text{Glucose} + 2\ P_i + 2\ \text{ADP} \longrightarrow 2\ \text{lactate} + 2\ \text{ATP} + 2\ H_2O$$

As in alcoholic fermentation, there is no net oxidation-reduction. The NADH formed in the oxidation of glyceraldehyde 3-phosphate is consumed in the reduction of pyruvate. *The regeneration of NAD + in the reduction of pyruvate to lactate or ethanol sustains the continued operation of glycolysis under anaerobic conditions.*

3. Only a fraction of the energy of glucose is released in its anaerobic conversion into ethanol or lactate. Much more energy can be extracted aerobically by means of the citric acid cycle and the electron-transport chain. The entry point to this oxidative pathway is *acetyl coenzyme A* (acetyl CoA), which is formed inside mitochondria by the oxidative decarboxylation of pyruvate.

$$\text{Pyruvate} + NAD^+ + \text{CoA} \longrightarrow \text{acetyl CoA} + CO_2 + \text{NADH}$$

This reaction, which is catalyzed by the pyruvate dehydrogenase complex, will be discussed in detail in Chapter 18. The NAD^+ required for this reaction and for the oxidation of glyceraldehyde 3-phosphate is regenerated when NADH ultimately transfers its electrons to O_2 through the electron-transport chain in mitochondria.

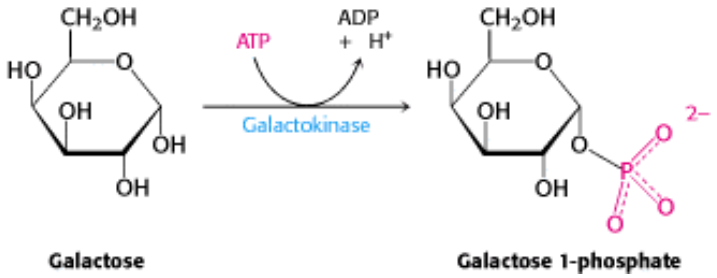
16.1.10. The Binding Site for NAD^+ Is Similar in Many Dehydrogenases

Although the enzymes taking part in glycolysis and the subsequent conversion of pyruvate are structurally diverse, the three dehydrogenases—glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase, and lactate dehydrogenase—have in common a domain for NAD^+ binding (Figure 16.13). This nucleotide-binding region is made up of four α helices and a sheet of six parallel β strands. Moreover, in all cases, the bound NAD^+ displays nearly the same conformation. This common structural domain, one of the first recurring structural domains to be discovered, is often called a *Rossmann fold* after Michael Rossmann, who first recognized it. This fold likely represents a primordial dinucleotide-binding domain that recurs in the dehydrogenases of glycolysis and other enzymes because of their descent from a common ancestor.

16.1.11. The Entry of Fructose and Galactose into Glycolysis

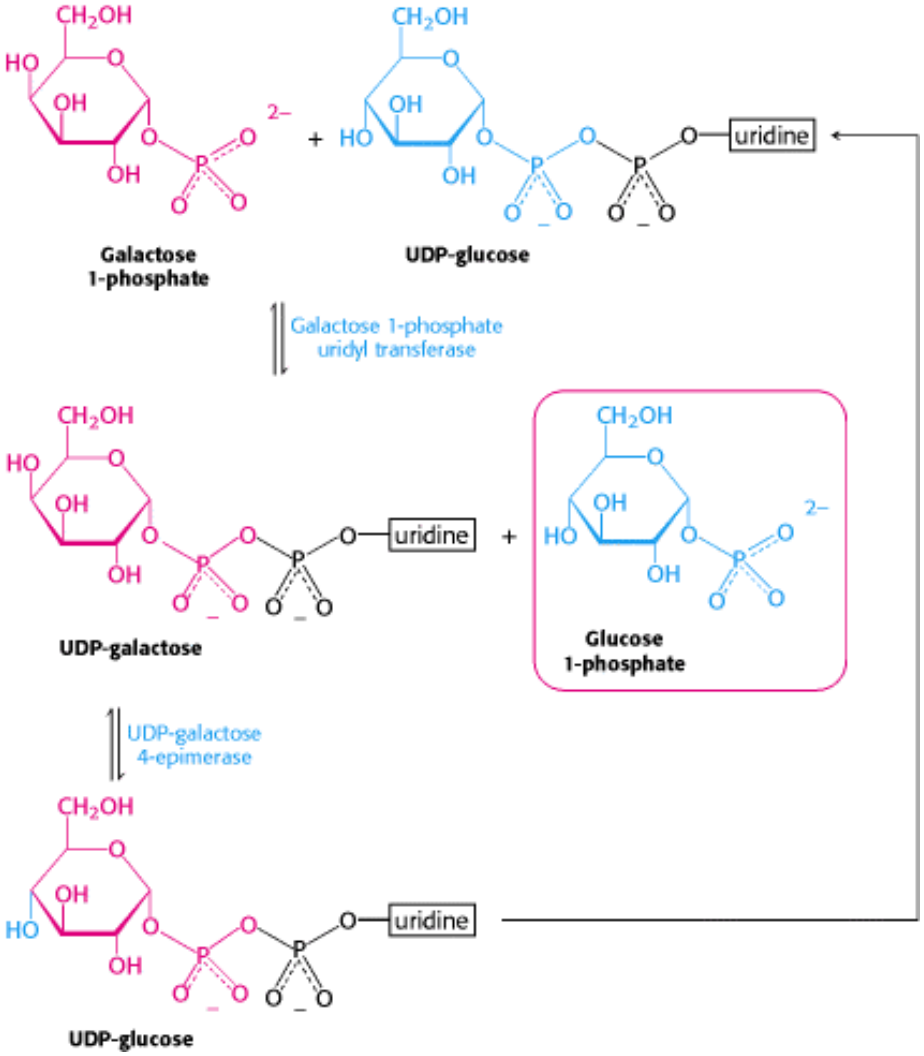
Although glucose is the most widely used monosaccharide, others also are important fuels. Let us consider how two abundant sugars—fructose and galactose—can be funneled into the glycolytic pathway (Figure 16.14). Much of the ingested fructose is metabolized by the liver, using the *fructose 1-phosphate pathway* (Figure 16.15). The first step is the phosphorylation of *fructose* to *fructose 1-phosphate* by *fructokinase*. Fructose 1-phosphate is then split into *glyceraldehyde* and *dihydroxyacetone phosphate*, an intermediate in glycolysis. This aldol cleavage is catalyzed by a specific *fructose 1-phosphate aldolase*. Glyceraldehyde is then phosphorylated to *glyceralde-hyde 3-phosphate*, a glycolytic intermediate, by *triose kinase*. Alternatively, *fructose can be phosphorylated to fructose 6-phosphate by hexokinase*. However, the affinity of hexokinase for glucose is 20 times as great as it is for fructose. Little fructose 6-phosphate is formed in the liver because glucose is so much more abundant in this organ. Moreover, glucose, as the preferred fuel, is also trapped in the muscle by the hexokinase reaction. Because liver and muscle phosphorylate glucose rather than fructose, adipose tissue is exposed to more fructose than glucose. Hence, the formation of fructose 6-phosphate is not competitively inhibited to a biologically significant extent, and most of the fructose in adipose tissue is metabolized through fructose 6-phosphate.

There are no catabolic pathways to metabolize *galactose*, so the strategy is to convert galactose into a metabolite of glucose. *Galactose* is converted into *glucose 6-phosphate* in four steps. The first reaction in the *galactose-glucose interconversion pathway* is the phosphorylation of galactose to galactose 1-phosphate by *galactokinase*.



Galactose **Galactose 1-phosphate**

Galactose 1-phosphate then acquires a uridyl group from uridine diphosphate glucose (UDP-glucose), an intermediate in the synthesis of glycosidic linkages (Section 21.4.2).



The products of this reaction, which is catalyzed by *galactose 1-phosphate uridyl transferase*, are UDP-galactose and glucose 1-phosphate. The galactose moiety of UDP-galactose is then epimerized to glucose. The configuration of the hydroxyl group at carbon 4 is inverted by *UDP-galactose 4-epimerase*.

The sum of the reactions catalyzed by galactokinase, the transferase, and the epimerase is:

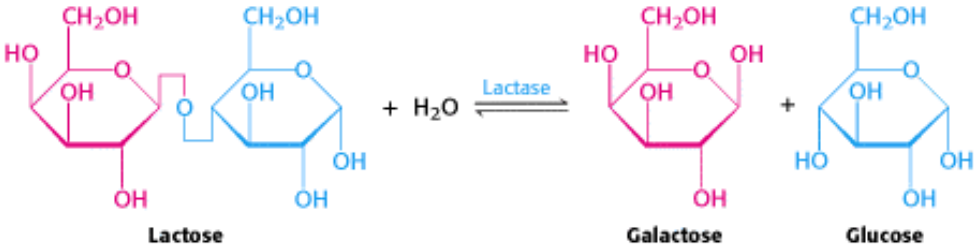
$$\text{Galactose} + \text{ATP} \longrightarrow \text{glucose 1-phosphate} + \text{ADP} + \text{H}^+$$

Note that UDP-glucose is not consumed in the conversion of galactose into glucose, because it is regenerated from UDP-galactose by the epimerase. This reaction is reversible, and the product of the reverse direction also is important. *The conversion of UDP-glucose into UDP-galactose is essential for the synthesis of galactosyl residues in complex polysaccharides and glycoproteins if the amount of galactose in the diet is inadequate to meet these needs.*

Finally, glucose 1-phosphate, formed from galactose, is isomerized to glucose 6-phosphate by *phosphoglucomutase*. We shall return to this reaction when we consider the synthesis and degradation of glycogen, which proceeds through glucose 1-phosphate, in Chapter 21.

16.1.12. Many Adults Are Intolerant of Milk Because They Are Deficient in Lactase

Many adults are unable to metabolize the milk sugar lactose and experience gastrointestinal disturbances if they drink milk. *Lactose intolerance*, or hypolactasia, is most commonly caused by a deficiency of the enzyme lactase, which cleaves lactose into glucose and galactose.



"Deficiency" is not quite the appropriate term, because a decrease in lactase is normal during development in all mammals. As children are weaned and milk becomes less prominent in their diets, lactase activity normally declines to about 5 to 10% of the level at birth. This decrease is not as pronounced with some groups of people, most notably Northern Europeans, and people from these groups can continue to ingest milk without gastrointestinal difficulties. With the appearance of milk-producing domesticated animals, a human being with a genetic alteration endowing high levels of lactase activity in adulthood would hypothetically have a selective advantage in being able to consume calories from the readily available milk.

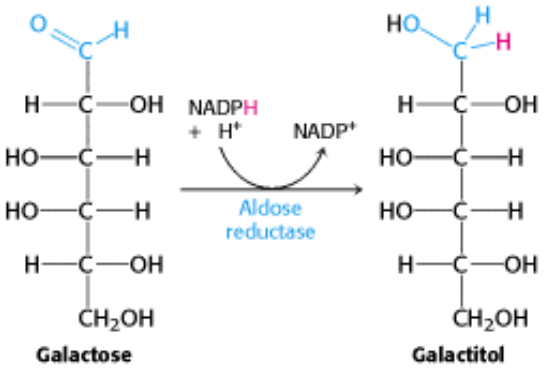
What happens to the lactose in the intestine of a lactase-deficient person? The lactose is a good energy source for microorganisms in the colon, and they ferment it to lactic acid while also generating methane (CH_4) and hydrogen gas (H_2). The gas produced creates the uncomfortable feeling of gut distention and the annoying problem of flatulence. The lactic acid produced by the microorganisms is osmotically active and draws water into the intestine, as does any undigested lactose, resulting in diarrhea. If severe enough, the gas and diarrhea hinder the absorption of other nutrients such as fats and proteins. The simplest treatment is to avoid the consumption of products containing much lactose. Alternatively, the enzyme lactase can be ingested with milk products.

16.1.13. Galactose Is Highly Toxic If the Transferase Is Missing

Less common than lactose intolerance are disorders that interfere with the metabolism of galactose. The disruption of galactose metabolism is referred to as *galactosemia*. The most common form, called classic galactosemia, is an inherited deficiency in galactose 1-phosphate uridyl transferase activity. Afflicted infants fail to thrive. They vomit or have diarrhea after consuming milk, and enlargement of the liver and jaundice are common, sometimes progressing to cirrhosis. Cataracts will form, and lethargy and retarded mental development also are common. The blood-galactose level is markedly elevated, and galactose is found in the urine. The absence of the transferase in red blood cells is a definitive diagnostic criterion.

The most common treatment is to remove galactose (and lactose) from the diet. The enigma of galactosemia is that, although elimination of galactose from the diet prevents liver disease and cataract development, the majority of patients still suffer from central nervous system malfunction, most commonly a delayed acquisition of language skills. Females will also display ovarian failure.

Cataract formation is better understood. A cataract is the clouding of the normally clear lens of the eye. If the transferase is not active in the lens of the eye, the presence of aldose reductase causes the accumulating galactose to be reduced to galactitol.



Galactitol is osmotically active, and water will diffuse into the lens, instigating the formation of cataracts. In fact, there is a high incidence of cataract formation with age in populations that consume substantial amounts of milk into adulthood.

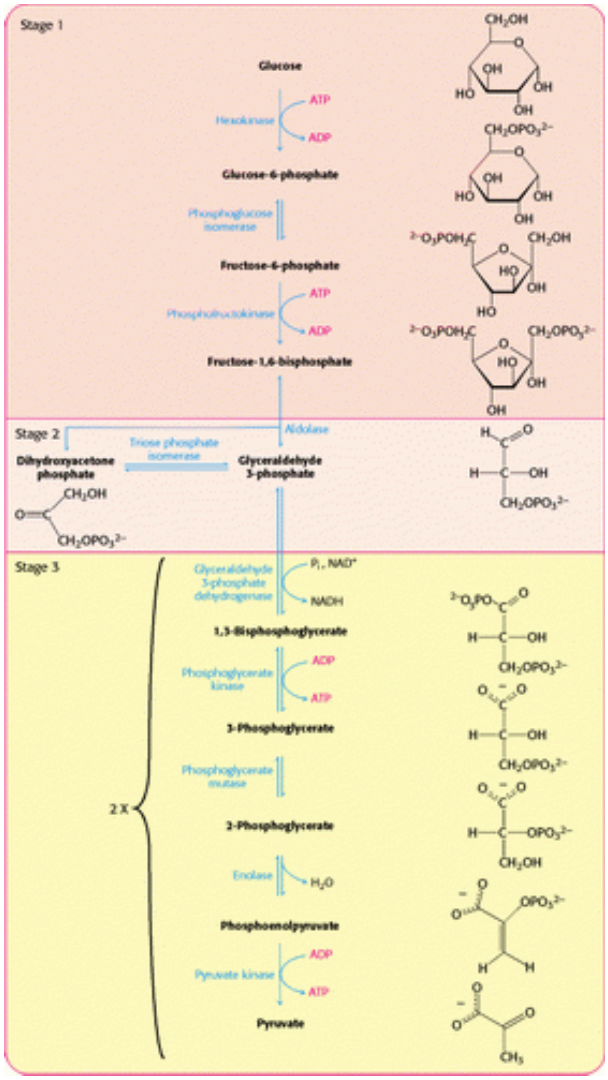
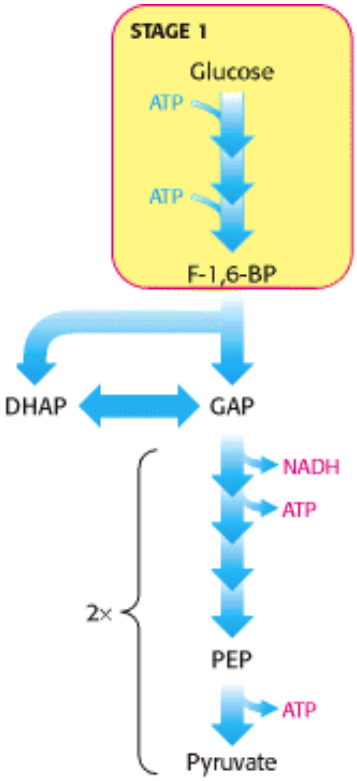


Figure 16.3. Stages of Glycolysis. The glycolytic pathway can be divided into three stages: (1) glucose is trapped and destabilized; (2) two interconvertible three-carbon molecules are generated by cleavage of six-carbon fructose; and (3) ATP is generated.



Stage 1 of glycolysis. The three steps of stage 1 begin with the phosphorylation of glucose by hexokinase.

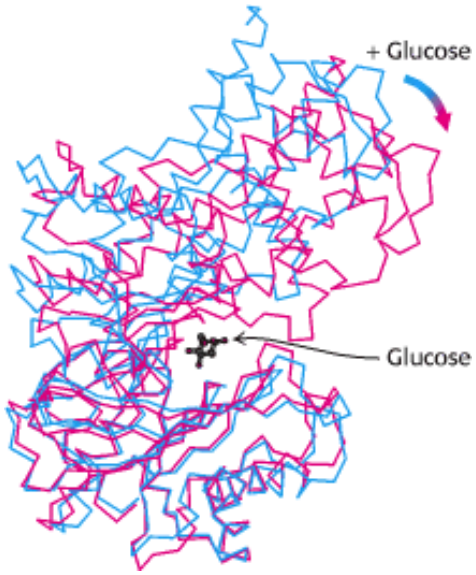
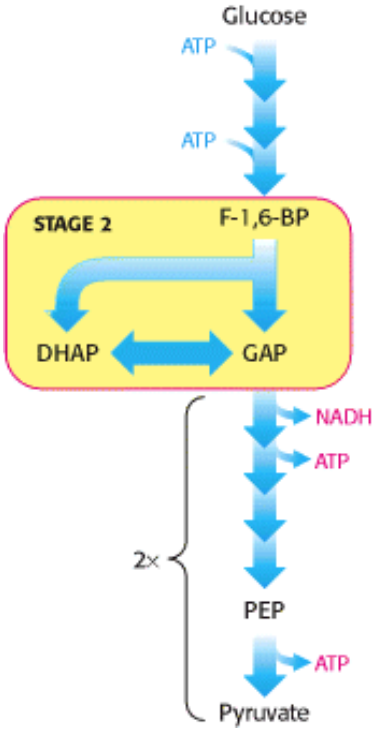


Figure 16.4. Induced Fit in Hexokinase. As shown in blue, the two lobes of hexokinase are separated in the absence of glucose. The conformation of hexokinase changes markedly on binding glucose, as shown in red. The two lobes of the enzyme come together and surround the substrate. [Courtesy of Dr. Thomas Steitz.]



Stage 2 of glycolysis. Two three-carbon fragments are produced from one six-carbon sugar.

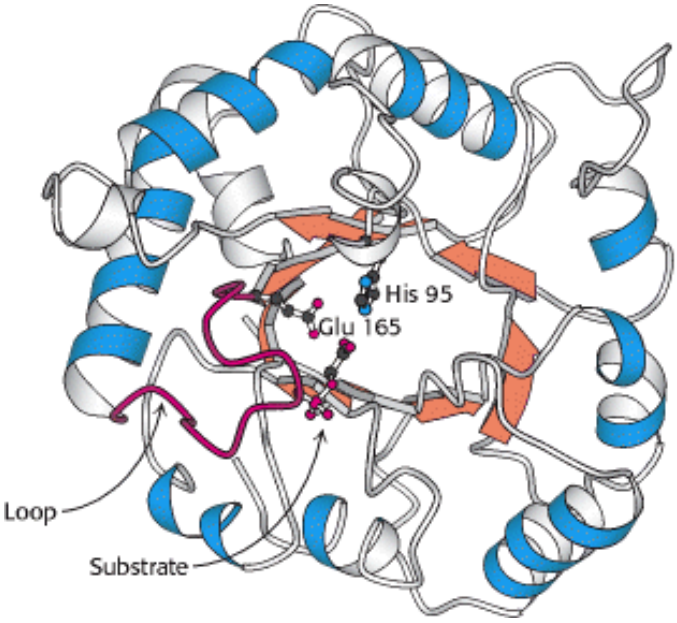


Figure 16.5. Structure of Triose Phosphate Isomerase. This enzyme consists of a central core of eight parallel β strands (orange) surrounded by eight α helices (blue). This structural motif, called an α β barrel, is also found in the glycolytic enzymes aldolase, enolase, and pyruvate kinase. Histidine 95 and glutamate 165, essential components of the active site of triose phosphate isomerase, are located in the barrel. A loop (red) closes off the active site on substrate binding.

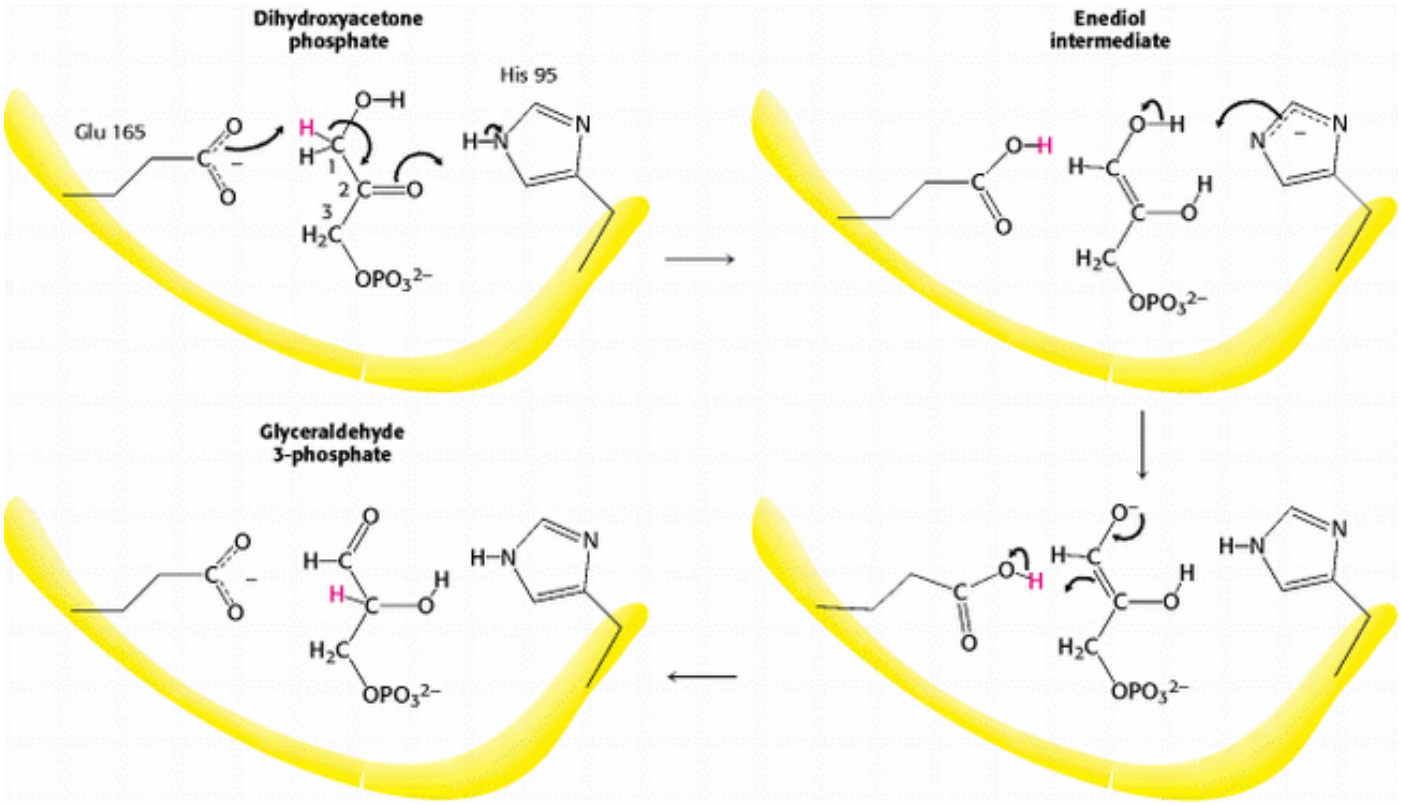
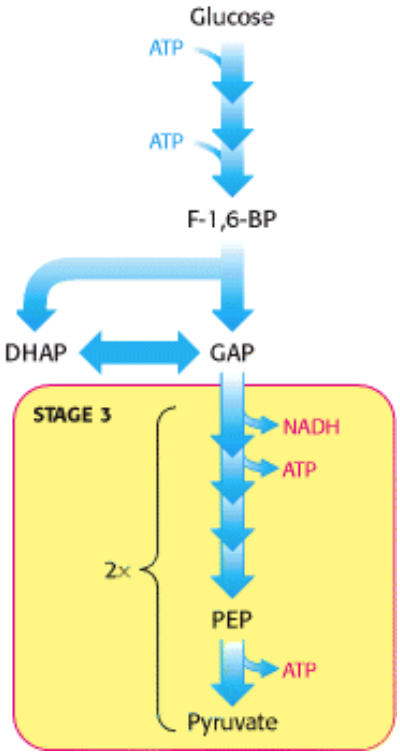


Figure 16.6. Catalytic Mechanism of Triose Phosphate Isomerase. Glutamate 165 transfers a proton between carbons with the assistance of histidine 95, which shuttles between the neutral and relatively rare negatively charged form. The latter is stabilized by interactions with other parts of the enzyme.



Stage 3 of Glycolysis. The oxidation of three-carbon fragments yields ATP.

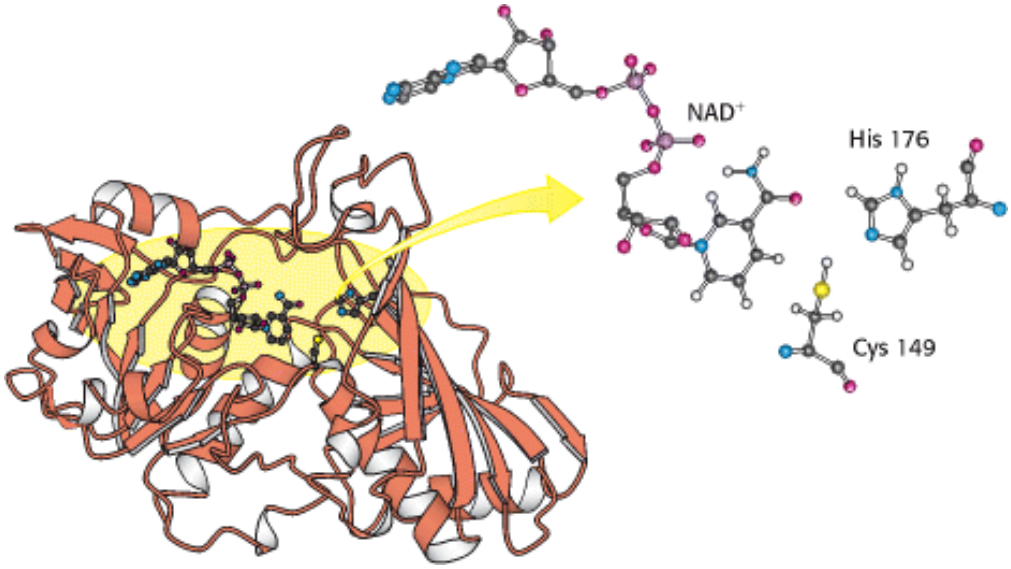


Figure 16.7. Structure of Glyceraldehyde 3-Phosphate Dehydrogenase. The active site includes a cysteine residue and a histidine residue adjacent to a bound NAD^+.

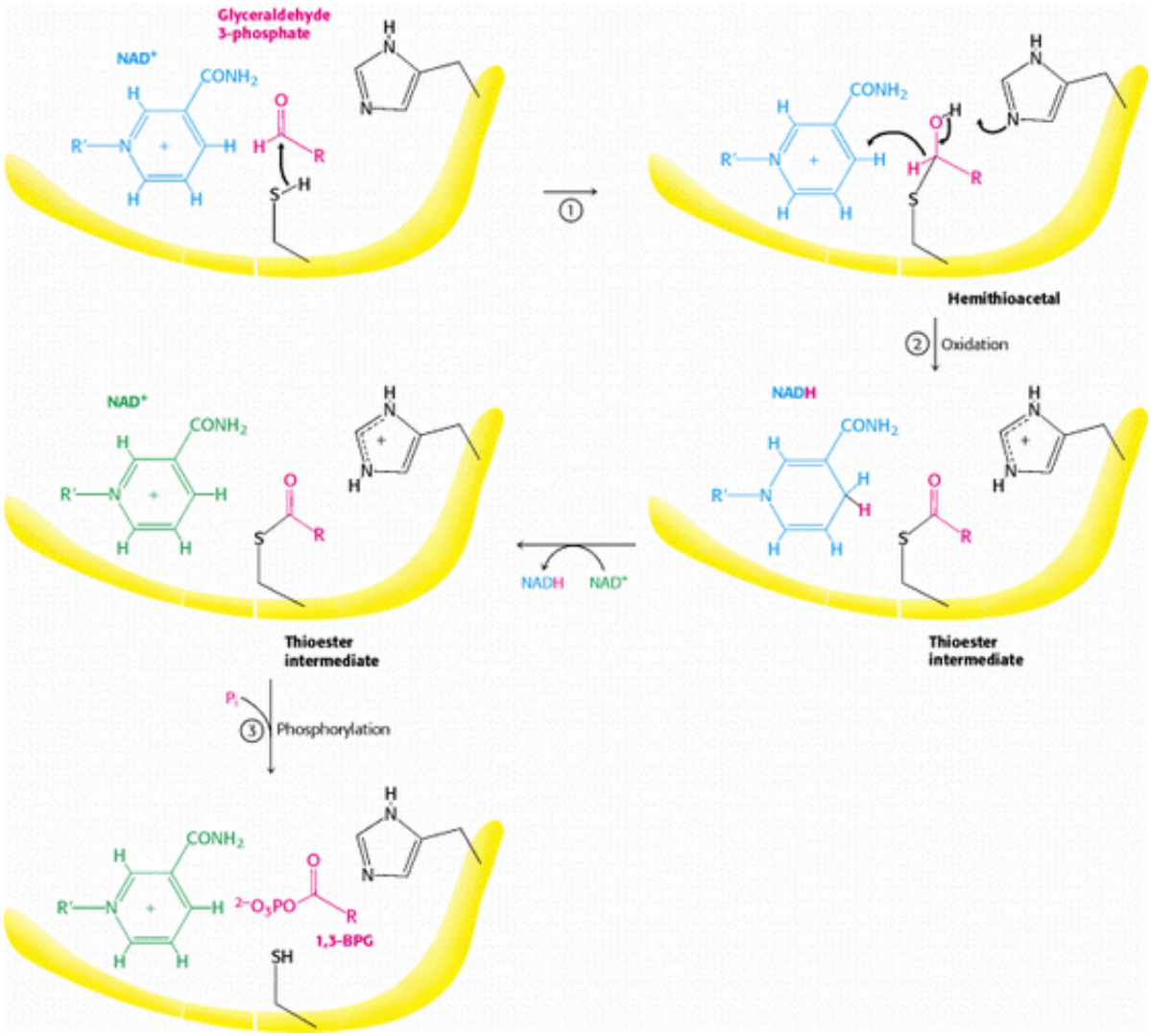


Figure 16.8. Catalytic Mechanism of Glyceraldehyde 3-Phosphate Dehydrogenase. The reaction proceeds through a

thioester intermediate, which allows the oxidation of glyceraldehyde to be coupled to the phosphorylation of 3-phosphoglycerate.

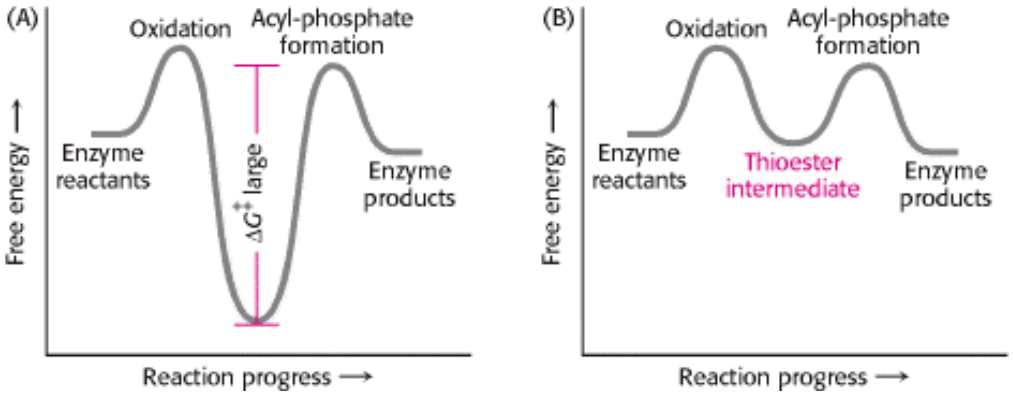


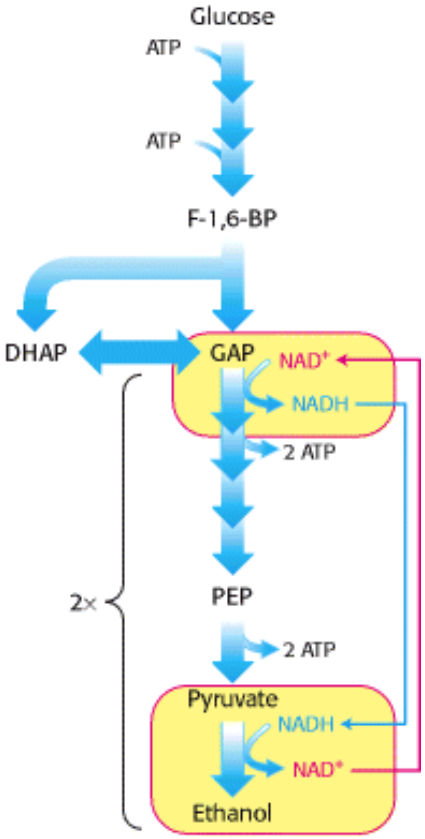
Figure 16.9. Free-Energy Profiles for Glyceraldehyde Oxidation Followed by Acyl-Phosphate Formation. (A) A hypothetical case with no coupling between the two processes. The second step must have a large activation barrier, making the reaction very slow. (B) The actual case with the two reactions coupled through a thioester intermediate.

Table 16.3. Reactions of glycolysis

Step	Reaction	Enzyme	Reaction type	$\Delta G^{\circ\prime}$ in kcal mol^{-1} (kJ mol^{-1})	ΔG in kcal mol^{-1} (kJ mol^{-1})
1	Glucose + ATP $\rightarrow$ glucose 6-phosphate + ADP + H^+	Hexokinase	Phosphoryl transfer	-4.0 (-16.7)	-8.0 (-33.5)
2	Glucose 6-phosphate $\rightleftharpoons$ fructose 6-phosphate	Phosphoglucose isomerase	Isomerization	+0.4 (+1.7)	-0.6 (-2.5)
3	Fructose 6-phosphate + ATP $\rightarrow$ fructose 1,6-bisphosphate + ADP + H^+	Phosphofructokinase	Phosphoryl transfer	-3.4 (-14.2)	-5.3 (-22.2)
4	Fructose 1,6-bisphosphate $\rightleftharpoons$ dihydroxyacetone phosphate + glyceraldehyde 3-phosphate	Aldolase	Aldol cleavage	+5.7 (+23.8)	-0.3 (-1.3)
5	Dihydroxyacetone phosphate $\rightleftharpoons$ glyceraldehyde 3-phosphate	Triose phosphate isomerase	Isomerization	+1.8 (+7.5)	+0.6 (+2.5)
6	Glyceraldehyde 3-phosphate + P_i + NAD^+ $\rightleftharpoons$ 1,3-bisphosphoglycerate + NADH + H^+	Glyceraldehyde 3-phosphate dehydrogenase	Phosphorylation coupled to oxidation	+1.5 (+6.3)	-0.4 (-1.7)
7	1,3-Bisphosphoglycerate + ADP $\rightleftharpoons$ 3-phosphoglycerate + ATP	Phosphoglycerate kinase	Phosphoryl transfer	-4.5 (-18.8)	+0.3 (+1.3)
8	3-Phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate	Phosphoglycerate mutase	Phosphoryl shift	+1.1 (+4.6)	+0.2 (+0.8)

9	2-Phosphoglycerate ⇌ phosphoenolpyruvate + H_2O	Enolase	Dehydration	+0.4 (+1.7)	-0.8 (-3.3)
10	Phosphoenolpyruvate + ADP + H^+ → pyruvate + ATP	Pyruvate kinase	Phosphoryl transfer	-7.5 (-31.4)	-4.0 (-16.7)

Note: Δ *G*, the actual free-energy change, has been calculated from Δ *G*°′ and known concentrations of reactants under typical physiologic conditions. Glycolysis can proceed only if the Δ *G* values of all reactions are negative. The small positive Δ *G* values of three of the above reactions indicate that the concentrations of metabolites in vivo in cells undergoing glycolysis are not precisely known.



Location of redox balance steps. The generation and consumption of NADH, located within the glycolytic pathway.

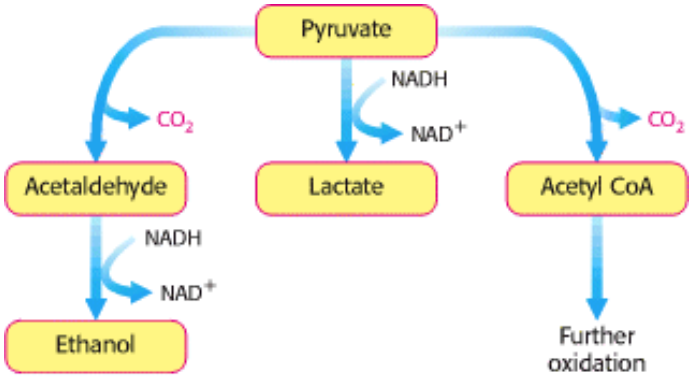


Figure 16.10. Diverse Fates of Pyruvate. Ethanol and lactate can be formed by reactions involving NADH. Alternatively, a two-carbon unit from pyruvate can be coupled to coenzyme A (see Section 17.1.1) to form acetyl CoA.

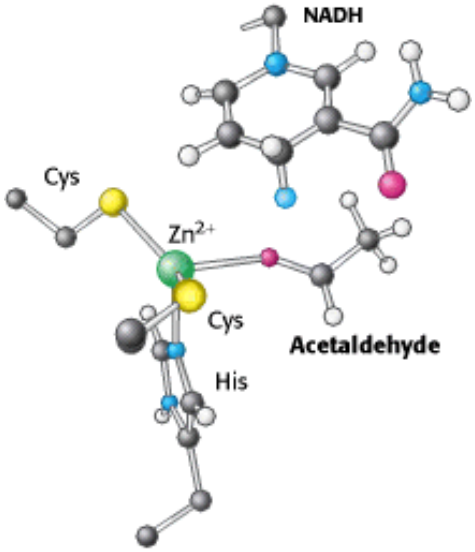


Figure 16.11. Active Site of Alcohol Dehydrogenase. The active site contains a zinc ion bound to two cysteine residues and one histidine residue. The zinc ion binds the acetaldehyde substrate through its oxygen atom, polarizing it so that it more easily accepts a hydride (light blue) from NADH. Only the nicotinamide ring of NADH is shown.

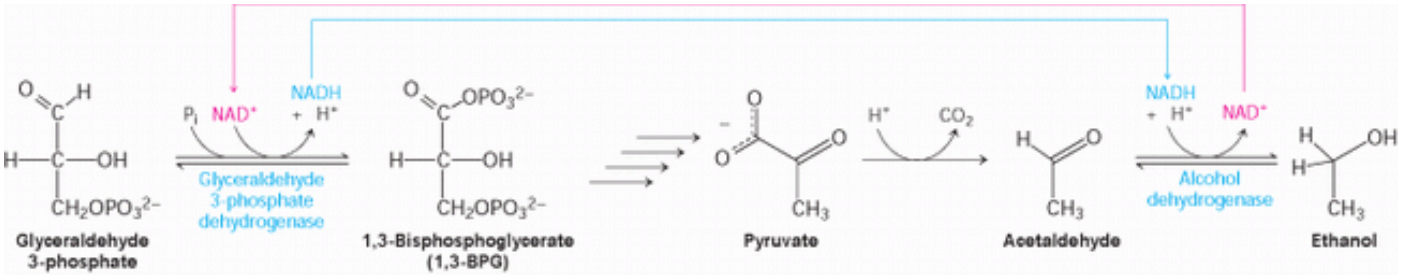


Figure 16.12. Maintaining Redox Balance. The NADH produced by the glyceraldehyde 3-phosphate dehydrogenase reaction must be reoxidized to NAD^+ for the glycolytic pathway to continue. In alcoholic fermentation, alcohol dehydrogenase oxidizes NADH and generates ethanol. In lactic acid fermentation (not shown), lactate dehydrogenase oxidizes NADH while generating lactic acid.

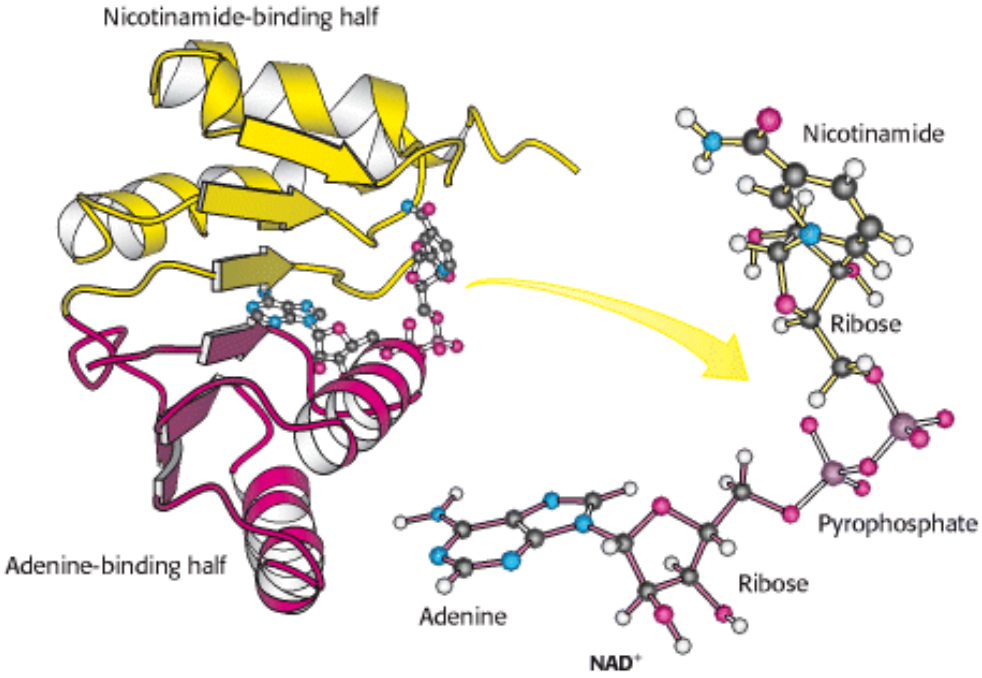


Figure 16.13. NAD$^+$ -binding region in dehydrogenases. The nicotinamide-binding half (yellow) is structurally similar to the adenine-binding half (red). The two halves together form a structural motif called a Rossmann fold. The NAD$^+$ molecule binds in an extended conformation.

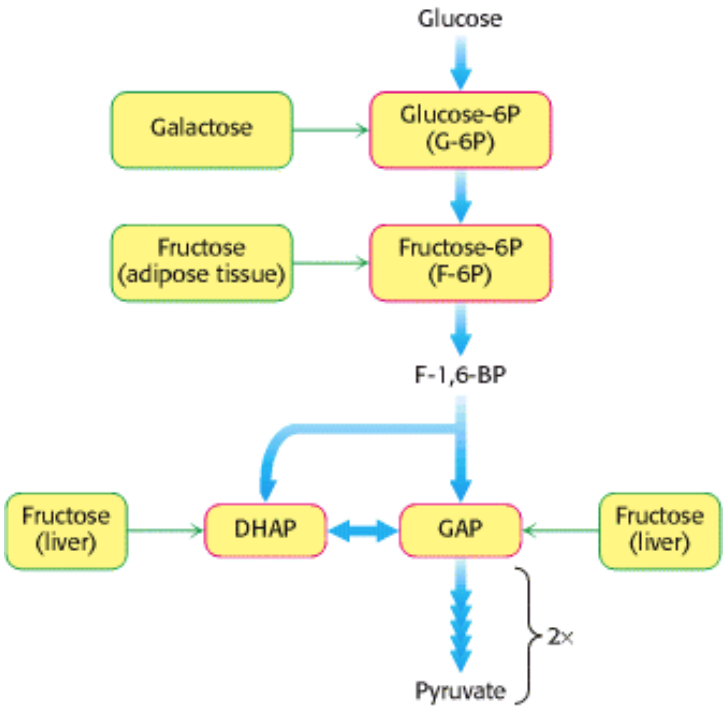


Figure 16.14. Entry Points in Glycolysis for Galactose and Fructose

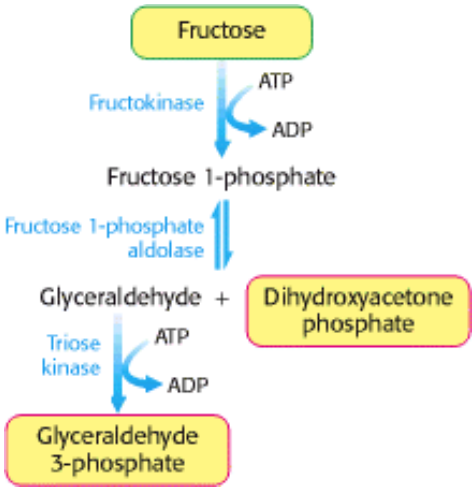
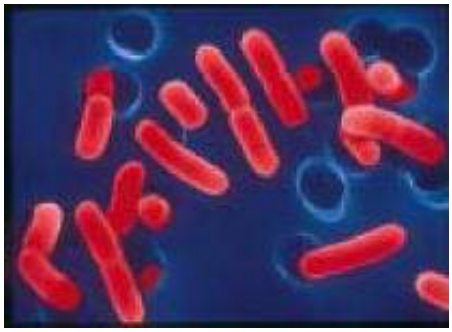


Figure 16.15. Fructose Metabolism. Fructose enters the glycolytic pathway in the liver through the fructose 1-phosphate pathway.



Scanning electron micrograph of *Lactobacillus*. The anaerobic bacteria *Lactobacillus* is shown here (artificially colored) at a magnification of 22, 245×. As suggested by its name, this genus of bacteria ferments glucose into lactic acid, and is widely used in the food industry. Lactobacillus is also a component of the normal human bacterial flora of the urogenital tract where, because of its ability to generate an acidic environment, it prevents growth of harmful bacteria. [Dr. Dennis Kunkel/PhotoTake.]

16.2. The Glycolytic Pathway Is Tightly Controlled

The flux through the glycolytic pathway must be adjusted in response to conditions both inside and outside the cell. The rate of conversion of glucose into pyruvate is regulated to meet two major cellular needs: (1) the production of ATP, generated by the degradation of glucose, and (2) the provision of building blocks for synthetic reactions, such as the formation of fatty acids. *In metabolic pathways, enzymes catalyzing essentially irreversible reactions are potential sites of control*. In glycolysis, the reactions catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase are virtually irreversible; hence, these enzymes would be expected to have regulatory as well as catalytic roles. In fact, each of them serves as a control site. Their activities are regulated by the reversible binding of allosteric effectors or by covalent modification. In addition, the amounts of these important enzymes are varied by the regulation of transcription to meet changing metabolic needs. The time required for reversible allosteric control, regulation by phosphorylation, and transcriptional control is typically in milliseconds, seconds, and hours, respectively.

16.2.1. Phosphofructokinase Is the Key Enzyme in the Control of Glycolysis

Phosphofructokinase is the most important control element in the mammalian glycolytic pathway (Figure 16.16). High

levels of ATP allosterically inhibit the enzyme in the liver (a 340-kd tetramer), thus lowering its affinity for fructose 6-phosphate. A high concentration of ATP converts the hyperbolic binding curve of fructose 6-phosphate into a sigmoidal one (Figure 16.17). ATP elicits this effect by binding to a specific regulatory site that is distinct from the catalytic site. AMP reverses the inhibitory action of ATP, and so *the activity of the enzyme increases when the ATP/AMP ratio is lowered*. In other words, *glycolysis is stimulated as the energy charge falls*. A fall in pH also inhibits phosphofructokinase activity. The inhibition of phosphofructokinase by H^+ prevents excessive formation of lactic acid (Section 16.1.9) and a precipitous drop in blood pH (acidosis).

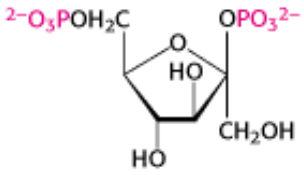
Why is AMP and not ADP the positive regulator of phosphofructokinase? When ATP is being utilized rapidly, the enzyme *adenylate kinase* (Section 9.4) can form ATP from ADP by the following reaction:

$$ADP + ADP \rightleftharpoons ATP + AMP$$

Thus, some ATP is salvaged from ADP, and AMP becomes the signal for the low-energy state. Moreover, the use of AMP as an allosteric regulator provides an especially sensitive control. We can understand why by considering, first, that the total adenylate pool ([ATP], [ADP], [AMP]) in a cell is constant over the short term and, second, that the concentration of ATP is greater than that of ADP and the concentration of ADP is, in turn, greater than that of AMP. Consequently, small-percentage changes in [ATP] result in larger-percentage changes in the concentrations of the other adenylate nucleotides. This magnification of small changes in [ATP] to larger changes in [AMP] leads to tighter control by increasing the range of sensitivity of phosphofructokinase.

Glycolysis also furnishes carbon skeletons for biosyntheses, and so a signal indicating whether building blocks are abundant or scarce should also regulate phosphofructokinase. Indeed, *phosphofructokinase is inhibited by citrate*, an early intermediate in the citric acid cycle (Section 17.1.3). A high level of citrate means that biosynthetic precursors are abundant and additional glucose should not be degraded for this purpose. Citrate inhibits phosphofructokinase by enhancing the inhibitory effect of ATP.

In 1980, *fructose 2,6-bisphosphate* (F-2,6-BP) was identified as a potent activator of phosphofructokinase. Fructose 2,6-bisphosphate activates phosphofructokinase by increasing its affinity for fructose 6-phosphate and diminishing the inhibitory effect of ATP (Figure 16.18). In essence, *Fructose* 2,6-*bisphosphate is an allosteric activator that shifts the conformational equilibrium of this tetrameric enzyme from the T state to the R state*.



Fructose 2,6-bisphosphate (F-2,6-BP)

16.2.2. A Regulated Bifunctional Enzyme Synthesizes and Degrades Fructose 2,6 -bisphosphate

How is the concentration of fructose 2,6-bisphosphate appropriately controlled? Two enzymes regulate the concentration of this important regulator of glycolysis by phosphorylating fructose 6-phosphate and dephosphorylating fructose 2,6-bisphosphate. Fructose 2,6-bisphosphate is formed in a reaction catalyzed by *phosphofructokinase 2* (*PFK2*), a different enzyme from phosphofructokinase. Fructose 2,6-bisphosphate is hydrolyzed to fructose 6-phosphate by a specific phosphatase, *fructose bisphosphatase 2* (*FBPase2*). The striking finding is that *both PFK2 and FBPase2 are present in a single 55-kd polypeptide chain* (Figure 16.19). This *bifunctional enzyme* contains an N-terminal *regulatory domain*, followed by a *kinase domain* and a *phosphatase domain*. PFK2 resembles adenylate kinase in having a P-loop NTPase domain (Sections 9.4.1 and 9.4.3), whereas FBPase2 resembles phosphoglycerate mutase (Section 16.1.7). Recall that the mutase is essentially a phosphatase. In the bifunctional enzyme, the phosphatase activity evolved to become specific

for F-2,6-BP. The bifunctional enzyme itself probably arose by the fusion of genes encoding the kinase and phosphatase domains.

The bifunctional enzyme exists in five isozymic forms (isoforms) that differ in size and kinetics as well as immunological and regulatory properties. Recall that isoenzymes, or isozymes, have essentially the same architectural plan and catalytic properties but differ in how they are regulated. The L isoform, which predominates in the liver, and the M isoform, found in muscle are generated by alternative splicing (Section 28.3.6) of the transcription product of a single gene. The L isoform helps to maintain blood-glucose homeostasis. In the liver, the concentration of fructose 6-phosphate rises when blood-glucose concentration is high, and the abundance of fructose 6-phosphate accelerates the synthesis of F-2,6-BP. Hence, *an abundance of fructose 6-phosphate leads to a higher concentration of F-2,6-BP, which in turn stimulates phosphofructokinase*. Such a process is called *feedforward stimulation*. What controls whether PFK2 or FBPase2 dominates the bifunctional enzyme's activities in the liver? The activities of PFK2 and FBPase2 are reciprocally controlled by *phosphorylation of a single serine residue*. When glucose is scarce, a rise in the blood level of the hormone glucagon triggers a cyclic AMP cascade, through its 7TM receptor and G_{α^s} (Section 15.1), leading to the phosphorylation of this bifunctional enzyme by protein kinase A (Figure 16.20). This covalent modification activates FBPase2 and inhibits PFK2, lowering the level of F-2,6-BP. Thus, glucose metabolism by the liver is curtailed. Conversely, when glucose is abundant, the enzyme loses its attached phosphate group. This covalent modification activates PFK2 and inhibits FBPase2, raising the level of F-2,6-BP and accelerating glycolysis. This coordinated control is facilitated by the location of the kinase and phosphatase domains on the same polypeptide chain as the regulatory domain. We shall return to this elegant switch when we consider the integration of carbohydrate metabolism (Section 16.4).

16.2.3. Hexokinase and Pyruvate kinase Also Set the Pace of Glycolysis

Phosphofructokinase is the most prominent regulatory enzyme in glycolysis, but it is not the only one. Hexokinase, the enzyme catalyzing the first step of glycolysis, is inhibited by its product, glucose 6-phosphate. High concentrations of this molecule signal that the cell no longer requires glucose for energy, for storage in the form of glycogen, or as a source of biosynthetic precursors, and the glucose will be left in the blood. For example, when phosphofructokinase is inactive, the concentration of fructose 6-phosphate rises. In turn, the level of glucose 6-phosphate rises because it is in equilibrium with fructose 6-phosphate. Hence, *the inhibition of phosphofructokinase leads to the inhibition of hexokinase*. However, the liver, in keeping with its role as monitor of blood-glucose levels, possesses a specialized isozyme of hexokinase called *glucokinase* that is not inhibited by glucose 6-phosphate. Glucokinase phosphorylates glucose only when it is abundant because it has about a 50-fold affinity for glucose than does hexokinase. The role of glucokinase is to provide glucose 6-phosphate for the synthesis of glycogen, a storage form of glucose (Section 21.4), and for the formation of fatty acids (Section 22.1). The low glucose affinity of glucokinase in the liver gives the brain and muscles first call on glucose when its supply is limited, whereas it ensures that glucose will not be wasted when it is abundant.

Why is phosphofructokinase rather than hexokinase the pacemaker of glycolysis? The reason becomes evident on noting that glucose 6-phosphate is not solely a glycolytic intermediate. Glucose 6-phosphate can also be converted into glycogen or it can be oxidized by the pentose phosphate pathway (Section 20.3) to form NADPH. The first irreversible reaction unique to the glycolytic pathway, the *committed step*, (Section 10.2), is the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. Thus, it is highly appropriate for phosphofructokinase to be the primary control site in glycolysis. In general, *the enzyme catalyzing the committed step in a metabolic sequence is the most important control element in the pathway*.

Pyruvate kinase, the enzyme catalyzing the third irreversible step in glycolysis, controls the outflow from this pathway. This final step yields ATP and pyruvate, a central metabolic intermediate that can be oxidized further or used as a building block. Several isozymic forms of pyruvate kinase (a tetramer of 57-kd subunits) encoded by different genes are present in mammals: the L type predominates in liver, and the M type in muscle and brain. The L and M forms of pyruvate kinase have many properties in common. Both bind phosphoenolpyruvate cooperatively. Fructose 1,6-bisphosphate, the product of the preceding irreversible step in glycolysis, activates both isozymes to enable them to keep pace with the oncoming high flux of intermediates. ATP allosterically inhibits both the L and the M forms of pyruvate

kinase to slow glycolysis when the energy charge is high. Finally, alanine (synthesized in one step from pyruvate, Section 24.2.2) also allosterically inhibits the pyruvate kinases—in this case, to signal that building blocks are abundant.

The isozymic forms differ in their susceptibility to covalent modification. The catalytic properties of the L form—but not of the M form—are also controlled by reversible phosphorylation (Figure 16.21). When the blood-glucose level is low, the glucagon-triggered cyclic AMP cascade (Section 15.1.5) leads to the phosphorylation of pyruvate kinase, which diminishes its activity. *These hormone-triggered phosphorylations, like that of the bifunctional enzyme controlling the levels of fructose* 2,6-*bisphosphate, prevent the liver from consuming glucose when it is more urgently needed by brain and muscle* (Section 30.3). We see here a clear-cut example of how isoenzymes contribute to the metabolic diversity of different organs. We will return to the control of glycolysis after considering gluconeogenesis.

16.2.4. A Family of Transporters Enables Glucose to Enter and Leave Animal Cells

Several glucose transporters mediate the thermodynamically downhill movement of glucose across the plasma membranes of animal cells. Each member of this protein family, named GLUT1 to GLUT5, consists of a single polypeptide chain about 500 residues long (Table 16.4). The common structural theme is the presence of 12 transmembrane segments (Figure 16.22).

The members of this family have distinctive roles:

1. GLUT1 and GLUT3, present in nearly all mammalian cells, are responsible for basal glucose uptake. Their K_M value for glucose is about 1 mM, significantly less than the normal serum-glucose level, which typically ranges from 4 mM to 8 mM. Hence, GLUT1 and GLUT3 continually transport glucose into cells at an essentially constant rate.

2. GLUT2, present in liver and pancreatic β cells, is distinctive in having a very high K_M value for glucose (15−20 mM). Hence, glucose enters these tissues at a biologically significant rate only when there is much glucose in the blood. The pancreas can thereby sense the glucose level and accordingly adjust the rate of insulin secretion. Insulin signals the need to remove glucose from the blood for storage as glycogen or conversion into fat (Section 30.3). The high K_M value of GLUT2 also ensures that glucose rapidly enters liver cells only in times of plenty.

3. GLUT4, which has a K_M value of 5 mM, transports glucose into muscle and fat cells. The presence of insulin, which signals the fed state, leads to a rapid increase in the number of GLUT4 transporters in the plasma membrane. Hence, insulin promotes the uptake of glucose by muscle and fat. The amount of this transporter present in muscle membranes increases in response to endurance exercise training.

4. GLUT5, present in the small intestine, functions primarily as a fructose transporter.

This family of transporters vividly illustrates how isoforms of a single protein can significantly shape the metabolic character of cells and contribute to their diversity and functional specialization. The transporters are members of a superfamily of transporters called the major facilitator (MF) superfamily. Members of this family transport sugars in organisms as diverse as *E. coli*, *Trypansoma brucei* (a parasitic protozoan that causes sleeping sickness), and human beings. An elegant solution to the problem of fuel transport evolved early and has been tailored to meet the needs of different organisms and even different tissues.

16.2.5. Cancer and Glycolysis

It has been known for decades that tumors display enhanced rates of glucose uptake and glycolysis. We now know that these enhanced rates of glucose processing are not fundamental to the development of cancer, but we can ask what selective advantage they might confer on cancer cells.

Cancer cells grow more rapidly than the blood vessels to nourish them; thus, as solid tumors grow, they are unable to obtain oxygen efficiently. In other words, they begin to experience *hypoxia*. Under these conditions, glycolysis leading to lactic acid fermentation becomes the primary source of ATP. Glycolysis is made more efficient in hypoxic tumors by the action of a transcription factor, *hypoxia-inducible transcription factor* (*HIF-1*). In the absence of oxygen, HIF-1 increases the expression of most glycolytic enzymes and the glucose transporters GLUT1 and GLUT3 (Table 16.5). In fact, glucose uptake correlates with tumor aggressiveness and a poor prognosis. These adaptations by the cancer cells enable the tumor to survive until vascularization can occur. HIF-1 also stimulates the growth of new tumors by increasing the expression of signal molecules, such as vascular endothelial growth factor (VEGF), that facilitate the growth of blood vessels (Figure 16.23). Without such vascularization, the tumor would cease to grow and either die or remain harmlessly small. Efforts are underway to develop drugs that inhibit vascularization of tumors.

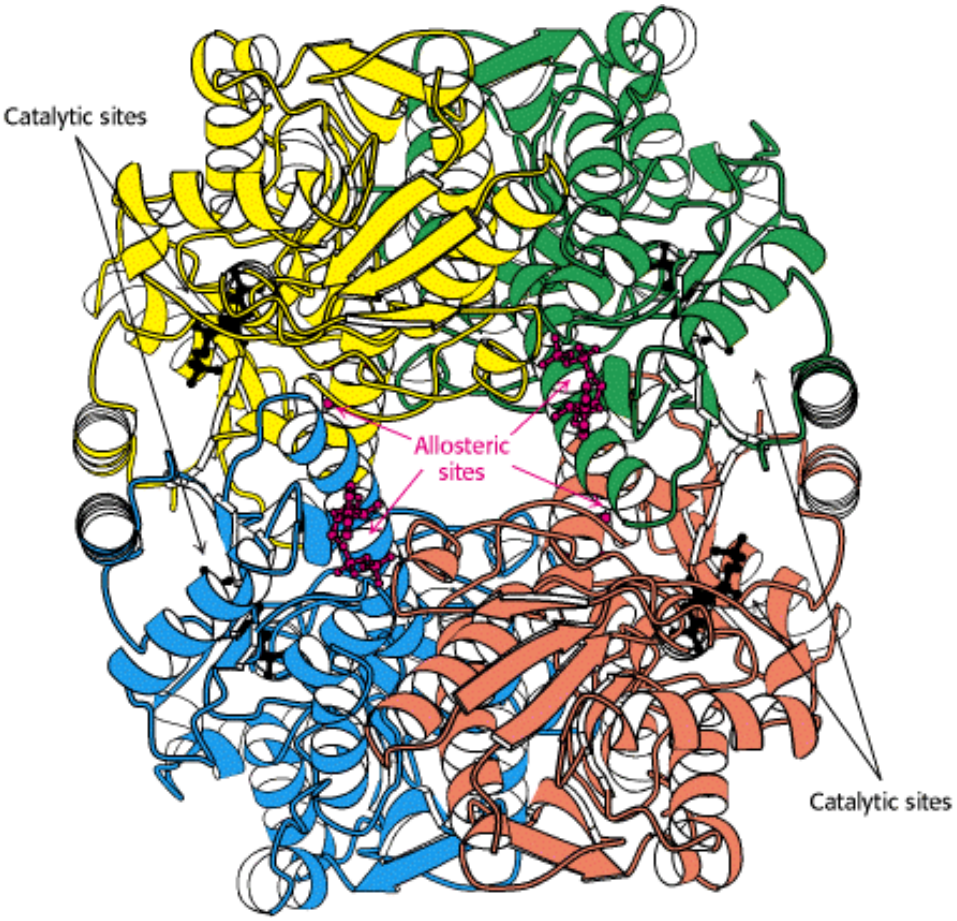


Figure 16.16. Structure of Phosphofructokinase. Phosphofructokinase in the liver is a tetramer of four identical subunits. The positions of the catalytic and allosteric sites are indicated.

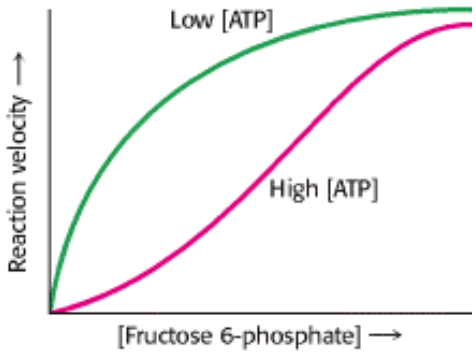


Figure 16.17. Allosteric Regulation of Phosphofructokinase. A high level of ATP inhibits the enzyme by decreasing its affinity for fructose 6-phosphate. AMP diminishes and citrate enhances the inhibitory effect of ATP.

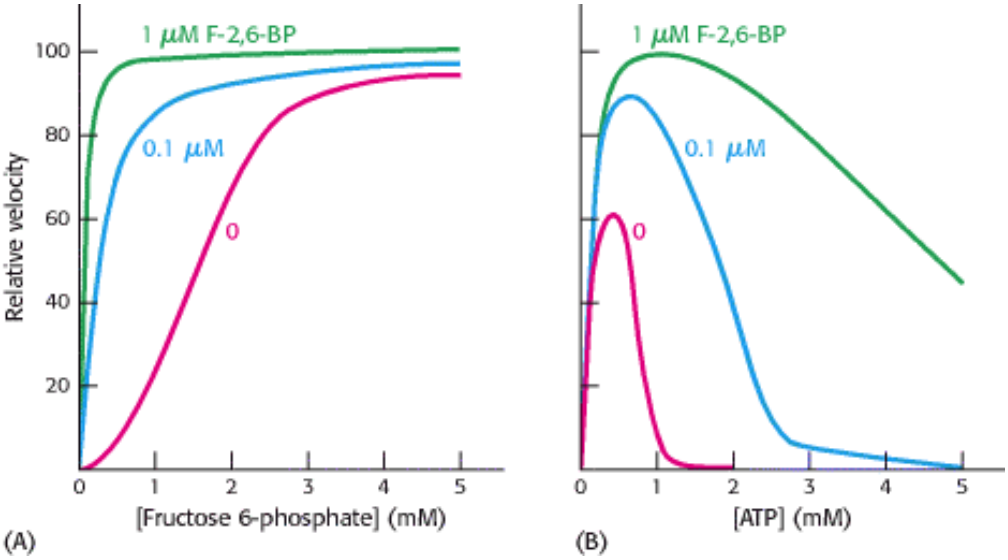


Figure 16.18. Activation of Phosphofructokinase by Fructose 2,6-Bisphosphate. (A) The sigmoidal dependence of velocity on substrate concentration becomes hyperbolic in the presence of 1 μM fructose 2,6-bisphosphate. (B) ATP, acting as a substrate, initially stimulates the reaction. As the concentration of ATP increases, it acts as an allosteric inhibitor. The inhibitory effect of ATP is reversed by fructose 2,6-bisphosphate. [After E. Van Schaftingen, M.F. Jett, L. Hue, and H. G. Hers. *Proc. Natl. Acad. Sci.* 78(1981):3483.]

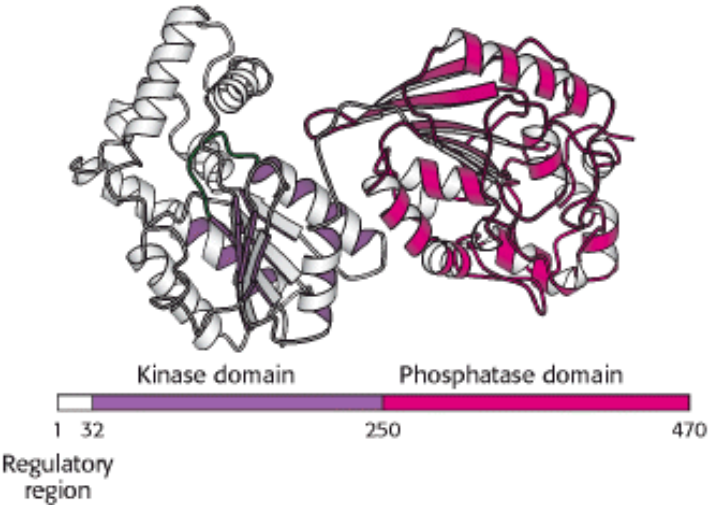


Figure 16.19. Domain Structure of the Bifunctional Enzyme Phosphofructokinase 2. The kinase domain (purple) is fused to the phosphatase domain (red). The kinase domain is a P-loop NTP hydrolase domain, as indicated by the purple shading (Section 9.4.4). The bar represents the amino acid sequence of the enzyme.

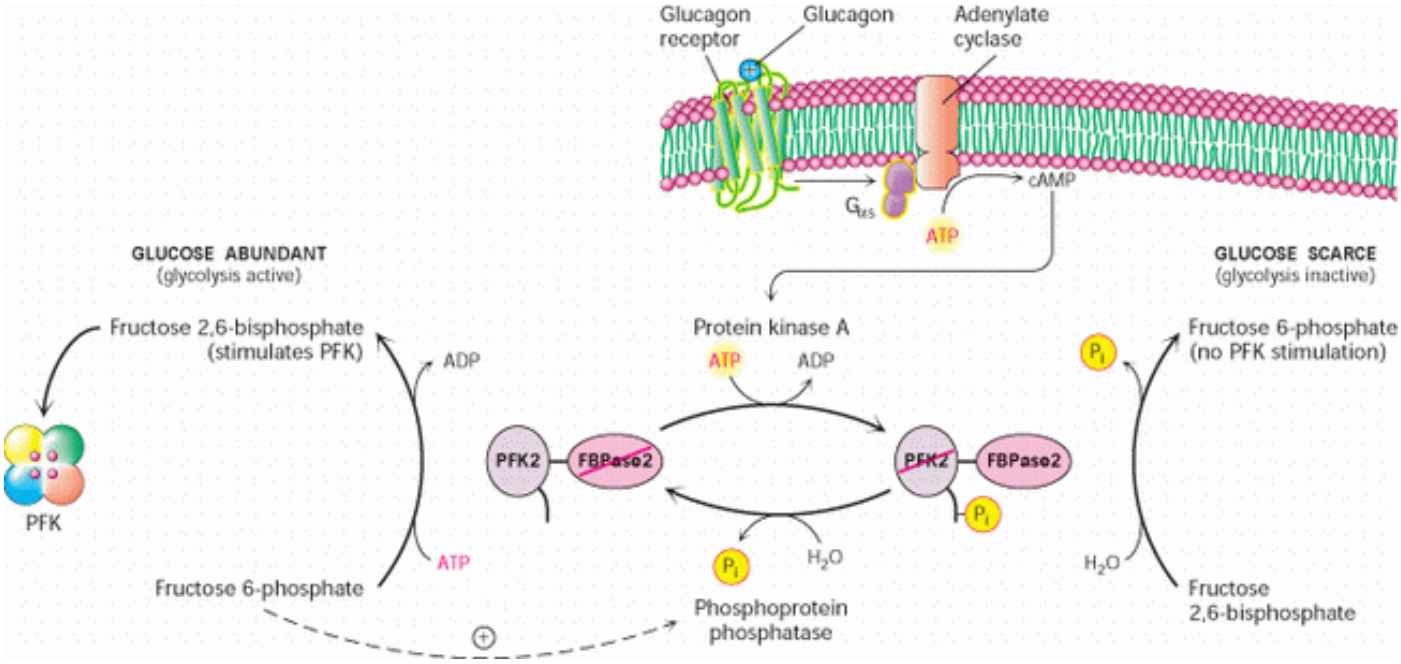


Figure 16.20. Control of the Synthesis and Degradation of Fructose 2,6-Bisphosphate. A low blood-glucose level as signaled by glucagon leads to the phosphorylation of the bifunctional enzyme and hence to a lower level of fructose 2,6-bisphosphate, slowing glycolysis. High levels of fructose 6-phosphate accelerate the formation of fructose 2,6-bisphosphate by facilitating the dephosphorylation of the bifunctional enzyme.

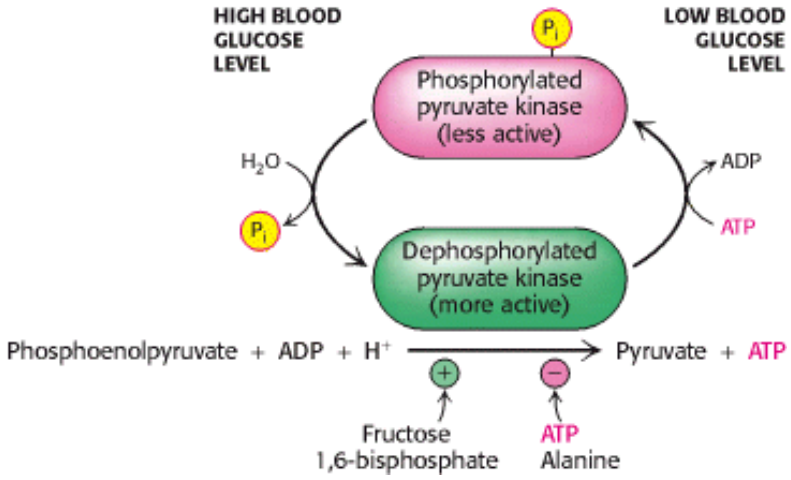


Figure 16.21. Control of the Catalytic Activity of Pyruvate Kinase. Pyruvate kinase is regulated by allosteric effectors and covalent modification.

Table 16.4. Family of glucose transporters

Name	Tissue location	K_m	Comments
GLUT1	All mammalian tissues	1 mM	Basal glucose uptake
GLUT2	Liver and pancreatic β cells	15 − 20 mM	In the pancreas, plays a role in regulation of insulin In the liver, removes excess glucose from the blood
GLUT3	All mammalian tissues	1 mM	Basal glucose uptake
GLUT4	Muscle and fat cells	5 mM	Amount in muscle plasma membrane increases with endurance training
GLUT5	Small intestine	—	Primarily a fructose transporter

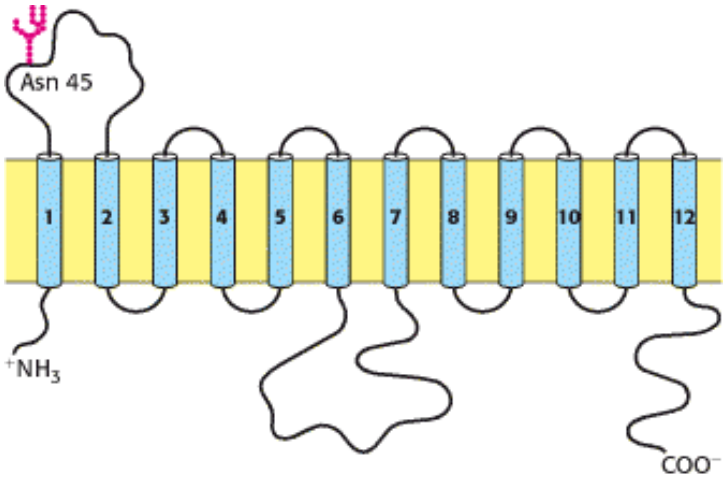


Figure 16.22. Model of a Mammalian Glucose Transporter. The hydrophobicity profile of the protein indicates 12 transmembrane α helices. [From M. Muekler, C. Caruso, S. A. Baldwin, M. Panico, M. Blench, H. R. Morris, W. J. Allard, G. E. Lienhard, and H. F. Lodish. *Science* 229(1985):941.]

Table 16.5. Proteins in glucose metabolism encoded by genes regulated by hypoxia-inducible factor

GLUT1
GLUT3
Hexokinase
Phosphofructokinase
Aldolase
Glyceraldehyde 3-phosphate dehydrogenase
Phosphoglycerate kinase
Enolase
Pyruvate kinase
Lactate dehydrogenase

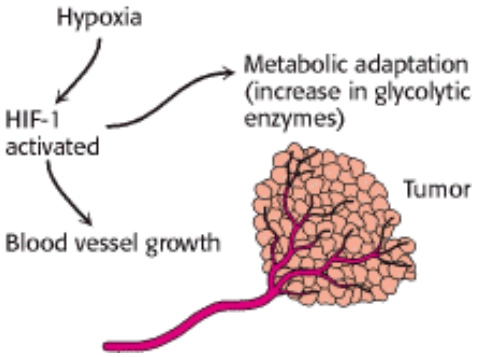
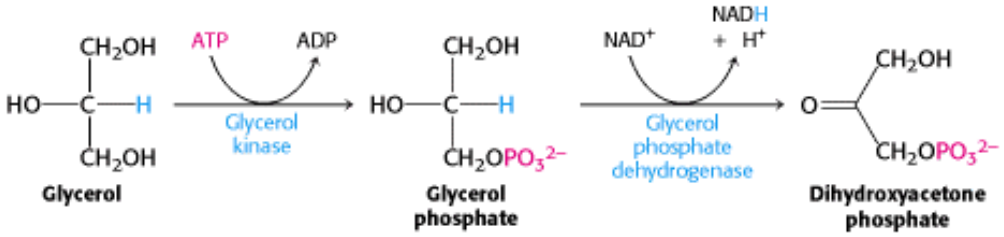


Figure 16.23. Alteration of Gene Expression in Tumors Due to Hypoxia. The hypoxic conditions inside a tumor mass lead to the activation of the hypoxia-inducible transcription factor (HIF-1), which induces metabolic adaptation (increase in glycolytic enzymes) and activates angiogenic factors that stimulate the growth of new blood vessels. [Adapted from C. V. Dang and G. L. Semenza. *Trends Biochem. Sci.* 24(1999):68−72.]

16.3. Glucose Can Be Synthesized from Noncarbohydrate Precursors

We now turn to the *synthesis of glucose from noncarbohydrate precursors*, a process called *gluconeogenesis*. This metabolic pathway is important because the brain depends on glucose as its primary fuel and red blood cells use only glucose as a fuel. The daily glucose requirement of the brain in a typical adult human being is about 120 g, which accounts for most of the 160 g of glucose needed daily by the whole body. The amount of glucose present in body fluids is about 20 g, and that readily available from glycogen, a storage form of glucose (Section 21.1), is approximately 190 g. Thus, the direct glucose reserves are sufficient to meet glucose needs for about a day. During a longer period of starvation, glucose must be formed from noncarbohydrate sources (Section 30.3.1).

The gluconeogenic pathway converts pyruvate into glucose. Noncarbohydrate precursors of glucose are first converted into pyruvate or enter the pathway at later intermediates such as oxaloacetate and dihydroxyacetone phosphate (Figure 16.24). The major noncarbohydrate precursors are *lactate*, *amino acids*, and *glycerol*. Lactate is formed by active skeletal muscle when the rate of glycolysis exceeds the rate of oxidative metabolism. Lactate is readily converted into pyruvate by the action of lactate dehydrogenase (Section 16.1.9). Amino acids are derived from proteins in the diet and, during starvation, from the breakdown of proteins in skeletal muscle (Section 30.3.1). The hydrolysis of triacylglycerols (Section 22.2.1) in fat cells yields glycerol and fatty acids. Glycerol is a precursor of glucose, but animals cannot convert fatty acids into glucose, for reasons that will be discussed later (Section 22.3.7). Glycerol may enter either the gluconeogenic or the glycolytic pathway at dihydroxyacetone phosphate.



The major site of gluconeogenesis is the *liver*, with a small amount also taking place in the *kidney*. Little gluconeogenesis takes place in the brain, skeletal muscle, or heart muscle. Rather, *gluconeogenesis in the liver and kidney helps to maintain the glucose level in the blood so that brain and muscle can extract sufficient glucose from it to meet their metabolic demands*.

16.3.1. Gluconeogenesis Is Not a Reversal of Glycolysis

In glycolysis, glucose is converted into pyruvate; in gluconeogenesis, pyruvate is converted into glucose. However, *gluconeogenesis is not a reversal of glycolysis*. Several reactions must differ because the equilibrium of glycolysis lies far on the side of pyruvate formation. The actual ΔG for the formation of pyruvate from glucose is about -20 kcal mol^{-1} (-84 kJ mol^{-1}) under typical cellular conditions. Most of the decrease in free energy in glycolysis takes place in the three essentially irreversible steps catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$
$$\Delta G = -8.0 \text{ kcal mol}^{-1} (-33 \text{ kJ mol}^{-1})$$

$$\text{Fructose 6-phosphate} + \text{ATP} \xrightarrow{\text{Phosphofructokinase}} \text{fructose 1,6-bisphosphate} + \text{ADP}$$
$$\Delta G = -5.3 \text{ kcal mol}^{-1} (-22 \text{ kJ mol}^{-1})$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{Pyruvate kinase}} \text{pyruvate} + \text{ATP}$$
$$\Delta G = -4.0 \text{ kcal mol}^{-1} (-17 \text{ kJ mol}^{-1})$$

In gluconeogenesis, the following new steps bypass these virtually irreversible reactions of glycolysis:

1. *Phosphoenolpyruvate is formed from pyruvate by way of oxaloacetate* through the action of pyruvate carboxylase and phosphoenolpyruvate carboxykinase.

$$\text{Pyruvate} + CO_2 + \text{ATP} + H_2O \xrightarrow{\text{Pyruvate carboxylase}} \text{oxaloacetate} + \text{ADP} + P_i + 2\,H^+$$

$$\text{Oxaloacetate} + \text{GTP} \xrightarrow{\text{Phosphoenolpyruvate carboxykinase}} \text{phosphoenolpyruvate} + \text{GDP} + CO_2$$

2. *Fructose 6-phosphate is formed from fructose 1,6-bisphosphate by hydrolysis of the phosphate ester at carbon 1*. Fructose 1,6-bisphosphatase catalyzes this exergonic hydrolysis.

$$\text{Fructose 1,6-bisphosphate} + H_2O \longrightarrow \text{fructose 6-phosphate} + P_i$$

3. *Glucose is formed by hydrolysis of glucose 6-phosphate* in a reaction catalyzed by glucose 6-phosphatase.

$$\text{Glucose 6-phosphate} + H_2O \longrightarrow \text{glucose} + P_i$$

We will examine each of these steps in turn.

16.3.2. The Conversion of Pyruvate into Phosphoenolpyruvate Begins with the Formation of Oxaloacetate

The first step in gluconeogenesis is the carboxylation of pyruvate to form oxaloacetate at the expense of a molecule of ATP. Then, oxaloacetate is decarboxylated and phosphorylated to yield phosphoenolpyruvate, at the expense of the high

phosphoryl-transfer potential of GTP. Both of these reactions take place inside the mitochondria.

$$\text{Pyruvate} + CO_2 + \text{ATP} + H_2O \rightleftharpoons \text{oxaloacetate} + \text{ADP} + P_i + 2\,H^+$$
$$\text{Oxaloacetate} + \text{GTP} \rightleftharpoons \text{phosphoenolpyruvate} + \text{GDP} + CO_2$$

The first reaction is catalyzed by *pyruvate carboxylase* and the second by *phosphoenolpyruvate carboxykinase*. The sum of these reactions is:

$$\text{Pyruvate} + \text{ATP} + \text{GTP} + H_2O \rightleftharpoons \text{phosphoenolpyruvate} + \text{ADP} + \text{GDP} + P_i + 2\,H^+$$

Pyruvate carboxylase is of special interest because of its structural, catalytic, and allosteric properties. The N-terminal 300 to 350 amino acids form an *ATP-grasp domain* (Figure 16.25), which is a widely used ATP-activating domain to be discussed in more detail when we investigate nucleotide biosynthesis (Section 25.1.1). The C-terminal 80 amino acids constitute a biotin-binding domain (Figure 16.26) that we will see again in fatty acid synthesis (Section 22.4.1). *Biotin* is a covalently attached prosthetic group, which serves as a *carrier of activated* CO_2. The carboxylate group of biotin is linked to the ε-amino group of a specific lysine residue by an amide bond (Figure 16.27). Note that biotin is attached to pyruvate carboxylase by a *long, flexible chain*.

The carboxylation of pyruvate takes place in three stages:

$$HCO_3^- + \text{ATP} \rightleftharpoons HOCO_2\text{-}PO_3^{2-} + \text{ADP}$$
$$\text{Biotin-enzyme} + HOCO_2\text{-}PO_3^{2-} \rightleftharpoons CO_2\text{-biotin-enzyme} + P_i$$
$$CO_2\text{-biotin-enzyme} + \text{pyruvate} \rightleftharpoons \text{biotin-enzyme} + \text{oxaloacetate}$$

Recall that, in aqueous solutions, CO_2 exists as HCO_3^- with the aid of carbonic anhydrase (Section 9.2). The HCO_3^- is activated to carboxyphosphate. This activated CO_2 is subsequently bonded to the N-1 atom of the biotin ring to form the carboxybiotin-enzyme intermediate (see Figure 16.27). The CO_2 attached to the biotin is quite activated. The $\Delta G^{\circ\prime}$ for its cleavage

$$CO_2\text{-biotin-enzyme} + H^+ \longrightarrow CO_2 + \text{biotin-enzyme}$$

is -4.7 kcal mol^{-1} (-20 kJ mol^{-1}). This negative $\Delta G^{\circ\prime}$ indicates that carboxybiotin is able to transfer CO_2 to acceptors without the input of additional free energy.

The activated carboxyl group is then transferred from carboxybiotin to pyruvate to form oxaloacetate. The long, flexible link between biotin and the enzyme enables this prosthetic group to rotate from one active site of the enzyme (the ATP-bicarbonate site) to the other (the pyruvate site).

The first partial reaction of pyruvate carboxylase, the formation of carboxybiotin, depends on the presence of acetyl CoA. *Biotin is not carboxylated unless acetyl CoA is bound to the enzyme.* Acetyl CoA has no effect on the second partial reaction. The allosteric activation of pyruvate carboxylase by acetyl CoA is an important physiological control mechanism that will be discussed in Section 17.3.1.

16.3.3. Oxaloacetate Is Shuttled into the Cytosol and Converted into Phosphoenolpyruvate

Pyruvate carboxylase is a mitochondrial enzyme, whereas the other enzymes of gluconeogenesis are cytoplasmic. Oxaloacetate, the product of the pyruvate carboxylase reaction, is reduced to malate inside the mitochondrion for transport to the cytosol. The reduction is accomplished by an NADH-linked malate dehydrogenase. When malate has been transported across the mitochondrial membrane, it is reoxidized to oxaloacetate by an NAD^+-linked malate dehydrogenase in the cytosol (Figure 16.28).

Finally, oxaloacetate is simultaneously *decarboxylated* and *phosphorylated* by phosphoenolpyruvate carboxykinase in the cytosol. The CO_2 that was added to pyruvate by pyruvate carboxylase comes off in this step. Recall that, in glycolysis, the presence of a phosphoryl group traps the unstable enol isomer of pyruvate as phosphoenolpyruvate (Section 16.1.7). In gluconeogenesis, the formation of the unstable enol is driven by decarboxylation—the oxidation of the carboxylic acid to CO_2 —and trapped by the addition of a phosphate to carbon 2 from GTP. The two-step pathway for the formation of phosphoenolpyruvate from pyruvate has a $\Delta G^{\circ\prime}$ of +0.2 kcal mol^{-1} (+0.13 kJ mol^{-1}) in contrast with +7.5 kcal mol^{-1} (+31 kJ mol^{-1}) for the reaction catalyzed by pyruvate kinase. The much more favorable $\Delta G^{\circ\prime}$ for the two-step pathway results from the use of a molecule of ATP to add a molecule of CO_2 in the carboxylation step that can be removed to power the formation of phosphoenolpyruvate in the decarboxylation step. *Decarboxylations often drive reactions otherwise highly endergonic*. This metabolic motif is used in the citric acid cycle (Section 17.1), the pentose phosphate pathway (Section 20.3.1), and fatty acid synthesis (Section 22.4.3).

16.3.4. The Conversion of Fructose 1,6-bisphosphate into Fructose 6-phosphate and Orthophosphate Is an Irreversible Step

On formation, phosphoenolpyruvate is metabolized by the enzymes of glycolysis but in the reverse direction. These reactions are near equilibrium under intracellular conditions; so, when conditions favor gluconeogenesis, the reverse reactions will take place until the next irreversible step is reached. This step is the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and P_i.

$$\text{Fructose 1,6-bisphosphate} + H_2O \xrightarrow{\text{Fructose 1,6-bisphosphatase}} \text{fructose 6-phosphate} + P_i$$

The enzyme responsible for this step is fructose 1,6-bisphosphatase. Like its glycolytic counterpart, it is an allosteric enzyme that participates in the regulation of gluconeogenesis. We will return to its regulatory properties later in the chapter.

16.3.5. The Generation of Free Glucose Is an Important Control Point

The fructose 6-phosphate generated by fructose 1,6-bisphosphatase is readily converted into glucose 6-phosphate. In most tissues, gluconeogenesis ends here. Free glucose is not generated; rather, the glucose 6-phosphate is processed in some other fashion, notably to form glycogen. One advantage to ending gluconeogenesis at glucose 6-phosphate is that, unlike free glucose, the molecule cannot diffuse out of the cell. To keep glucose inside the cell, the generation of free glucose is controlled in two ways. First, the enzyme responsible for the conversion of glucose 6-phosphate into glucose, *glucose 6-phosphatase*, is regulated. Second, the enzyme is present only in tissues whose metabolic duty is to maintain blood-glucose homeostasis—tissues that release glucose into the blood. These tissues are the liver and to a lesser extent the kidney.

This final step in the generation of glucose does not take place in the cytosol. Rather, glucose 6-phosphate is transported into the lumen of the endoplasmic reticulum, where it is hydrolyzed to glucose by glucose 6-phosphatase, which is bound to the membrane (Figure 16.29). An associated Ca^{2+}-binding stabilizing protein is essential for phosphatase activity. Glucose and P_i are then shuttled back to the cytosol by a pair of transporters. The glucose transporter in the endoplasmic reticulum membrane is like those found in the plasma membrane (Section 16.2.4). It is striking that five proteins are needed to transform cytosolic glucose 6-phosphate into glucose.

16.3.6. Six High Transfer Potential Phosphoryl Groups Are Spent in Synthesizing Glucose from Pyruvate

Conceptual Insights, Energetics of Glucose Metabolism. See the section on gluconeogenesis in the Conceptual Insights module to review why and how gluconeogenesis must differ from the reversal of glycolysis.

The stoichiometry of gluconeogenesis is:

$$\begin{aligned} 2\ \text{Pyruvate} + 4\ \text{ATP} + 2\ \text{GTP} + 2\ \text{NADH} + 6\ \text{H}_2\text{O} \longrightarrow \\ \text{glucose} + 4\ \text{ADP} + 2\ \text{GDP} + 6\ \text{P}_\text{i} + 2\ \text{NAD}^+ + 2\ \text{H}^+ \\ \Delta G^{\circ\prime} = -9\ \text{kcal mol}^{-1}\ (-38\ \text{kJ mol}^{-1}) \end{aligned}$$

In contrast, the stoichiometry for the reversal of glycolysis is:

$$\begin{aligned} 2\ \text{Pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}_2\text{O} \longrightarrow \\ \text{glucose} + 2\ \text{ADP} + 2\ \text{P}_\text{i} + 2\ \text{NAD}^+ \\ \Delta G^{\circ\prime} = +20\ \text{kcal mol}^{-1}\ (+84\ \text{kJ mol}^{-1}) \end{aligned}$$

Note that *six* nucleotide triphosphate molecules are hydrolyzed to synthesize glucose from pyruvate in gluconeogenesis, whereas only *two* molecules of ATP are generated in glycolysis in the conversion of glucose into pyruvate. Thus, the extra cost of gluconeogenesis is four high phosphoryl-transfer potential molecules per molecule of glucose synthesized from pyruvate. The four additional high phosphoryl-transfer potential molecules are needed to turn an energetically unfavorable process (the reversal of glycolysis, $\Delta G^{\circ\prime}$ = + 20 kcal mol^{-1} [+84 kJ mol^{-1}]) into a favorable one (gluconeogenesis, $\Delta G^{\circ\prime}$ = -9 kcal mol^{-1} [-38 kJ mol^{-1}]). This is a clear example of the coupling of reactions: ATP hydrolysis is used to power an energetically unfavorable reaction.

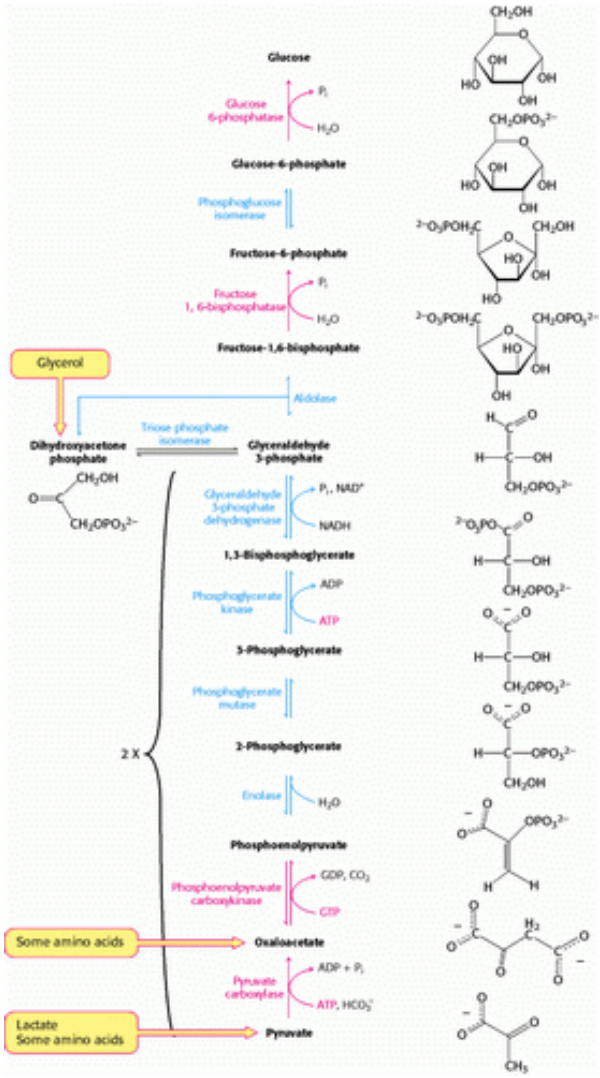


Figure 16.24. Pathway of Gluconeogenesis. The distinctive reactions and enzymes of this pathway are shown in red. The other reactions are common to glycolysis. The enzymes for gluconeogenesis are located in the cytosol, except for pyruvate carboxylase (in the mitochondria) and glucose 6-phosphatase (membrane bound in the endoplasmic reticulum). The entry points for lactate, glycerol, and amino acids are shown.

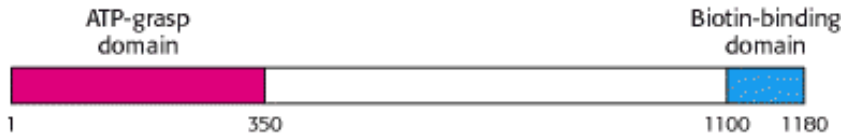


Figure 16.25. Domain Structure of Pyruvate Carboxylase. The ATP-grasp domain activates HCO_3^- and transfers CO_2 to the biotin-binding domain. From there, the CO_2 is transferred to pyruvate generated in the central domain.

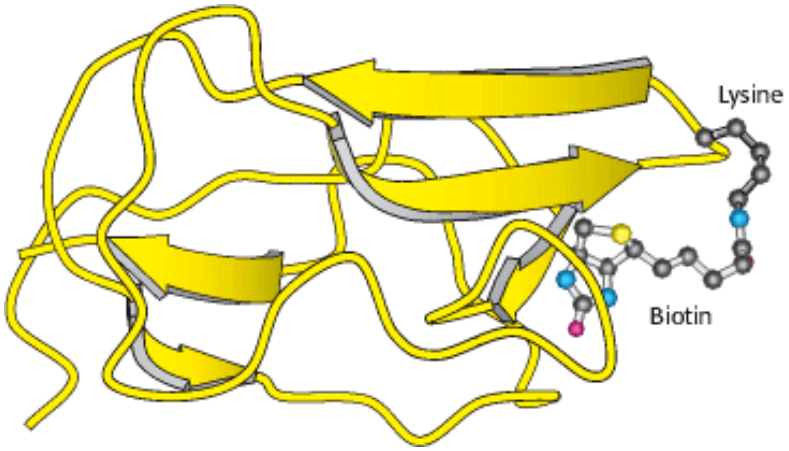


Figure 16.26. Biotin-Binding Domain of Pyruvate Carboxylase. This likely structure is based on the structure of the homologous domain from the enzyme acetyl CoA carboxylase (Section 22.4.1). The biotin is on a flexible tether, allowing it to move between the ATP-bicarbonate site and the pyruvate site.

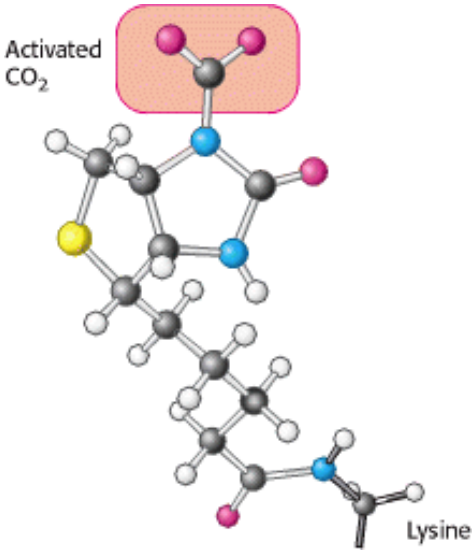


Figure 16.27. Structure of Carboxybiotin.

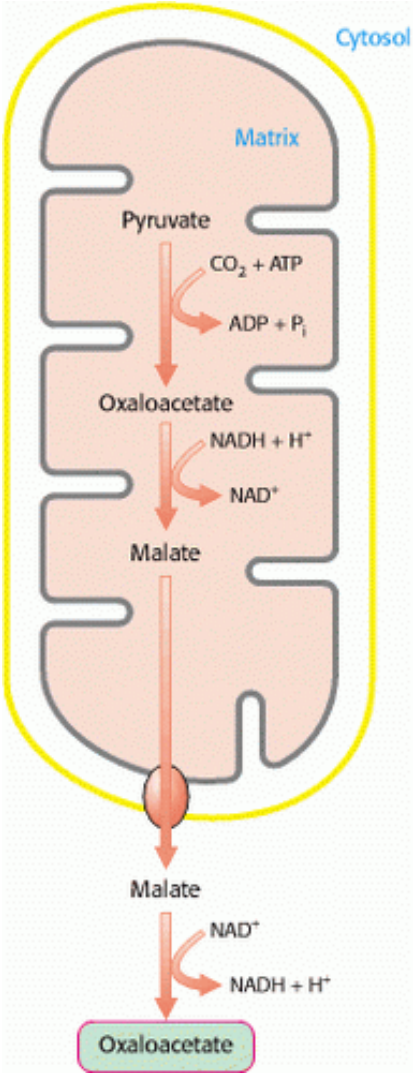


Figure 16.28. Compartmental Cooperation. Oxaloacetate utilized in the cytosol for gluconeogenesis is formed in the mitochondrial matrix by carboxylation of pyruvate. Oxaloacetate leaves the mitochondrion by a specific transport system (not shown) in the form of malate, which is reoxidized to oxaloacetate in the cytosol.

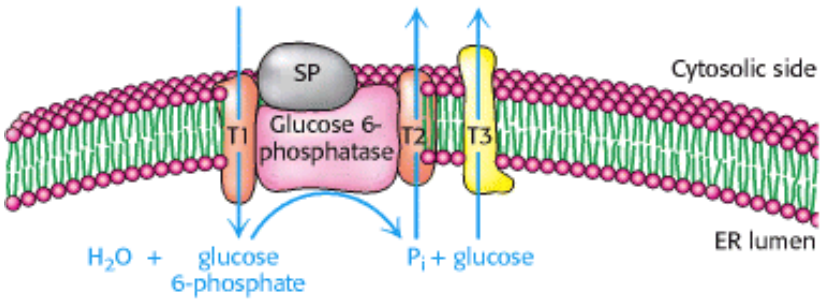


Figure 16.29. Generation of Glucose from Glucose 6-Phosphate. Several endoplasmic reticulum (ER) proteins play a role in the generation of glucose from glucose 6-phosphate. T1 transports glucose 6-phosphate into the lumen of the ER, whereas T2 and T3 transport Pi and glucose, respectively, back into the cytosol. Glucose 6-phosphatase is stabilized by a Ca^{2+}-binding protein (SP). [After A. Buchell and I. D. Waddel. *Biochem. Biophys. Acta* 1092(1991):129.]

16.4. Gluconeogenesis and Glycolysis Are Reciprocally Regulated

Gluconeogenesis and glycolysis are coordinated so that within a cell one pathway is relatively inactive while the other is highly active. If both sets of reactions were highly active at the same time, the net result would be the hydrolysis of four nucleotide triphosphates (two ATP plus two GTP) per reaction cycle. Both glycolysis and gluconeogenesis are highly exergonic under cellular conditions, and so there is no thermodynamic barrier to such simultaneous activity. However, the *amounts* and *activities* of the distinctive enzymes of each pathway are controlled so that both pathways are not highly active at the same time. The rate of glycolysis is also determined by the concentration of glucose, and the rate of gluconeogenesis by the concentrations of lactate and other precursors of glucose.

The interconversion of fructose 6-phosphate and fructose 1,6-bisphosphate is stringently controlled (Figure 16.30). As discussed in Section 16.2.1, AMP stimulates phosphofructokinase, whereas ATP and citrate inhibit it. Fructose 1,6-bisphosphatase, on the other hand, is inhibited by AMP and activated by citrate. A high level of AMP indicates that the energy charge is low and signals the need for ATP generation. Conversely, high levels of ATP and citrate indicate that the energy charge is high and that biosynthetic intermediates are abundant. Under these conditions, glycolysis is nearly switched off and gluconeogenesis is promoted.

Phosphofructokinase and fructose 1,6-bisphosphatase are also reciprocally controlled by *fructose 2,6-bisphosphate in the liver* (Section 16.2.2). The level of F-2,6-BP is low during starvation and high in the fed state, because of the antagonistic effects of glucagon and insulin on the production and degradation of this signal molecule. *Fructose 2,6-bisphosphate strongly stimulates phosphofructokinase and inhibits fructose 1,6-bisphosphatase*. Hence, glycolysis is accelerated and gluconeogenesis is diminished in the fed state. During starvation, gluconeogenesis predominates because the level of F-2,6-BP is very low. Glucose formed by the liver under these conditions is essential for the viability of brain and muscle.

The interconversion of phosphoenolpyruvate and pyruvate also is precisely regulated. Recall that pyruvate kinase is controlled by allosteric effectors and by phosphorylation (Section 16.2.3). High levels of ATP and alanine, which signal that the energy charge is high and that building blocks are abundant, inhibit the enzyme in liver. Conversely, pyruvate carboxylase, which catalyzes the first step in gluconeogenesis from pyruvate, is activated by acetyl CoA and inhibited by ADP. Likewise, ADP inhibits phosphoenolpyruvate carboxykinase. Hence, gluconeogenesis is favored when the cell is rich in biosynthetic precursors and ATP.

The amounts and the activities of these essential enzymes also are regulated. The regulators in this case are hormones. Hormones affect gene expression primarily by changing the rate of transcription, as well as by regulating the degradation of mRNA. Insulin, which rises subsequent to eating, stimulates the expression of phosphofructokinase, pyruvate kinase, and the bifunctional enzyme that makes and degrades F-2,6-BP. Glucagon, which rises during starvation, inhibits the expression of these enzymes and stimulates instead the production of two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and fructose 1,6-bisphosphatase. Transcriptional control in eukaryotes is much slower than allosteric control; it takes hours or days in contrast with seconds to minutes. The richness and complexity of hormonal control are graphically displayed by the promoter of the phosphoenolpyruvate carboxykinase gene, which contains regulatory sequences that respond to insulin, glucagon, glucocorticoids, and thyroid hormone (Figure 16.31).

16.4.1. Substrate Cycles Amplify Metabolic Signals and Produce Heat

A pair of reactions such as the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate and its hydrolysis back to fructose 6-phosphate is called a *substrate cycle*. As already mentioned, both reactions are not simultaneously fully active in most cells, because of reciprocal allosteric controls. However, the results of isotope-labeling studies have shown that some fructose 6-phosphate is phosphorylated to fructose 1,6-bisphosphate in gluconeogenesis. There also is a limited degree of cycling in other pairs of opposed irreversible reactions. This cycling was regarded as an imperfection in metabolic control, and so substrate cycles have sometimes been called *futile cycles*. Indeed, there are pathological conditions, such as malignant hyperthermia, in which control is lost and both pathways proceed rapidly with the

concomitant generation of heat by the rapid, uncontrolled hydrolysis of ATP.

Despite such extraordinary circumstances, it now seems likely that substrate cycles are biologically important. One possibility is that *substrate cycles amplify metabolic signals*. Suppose that the rate of conversion of A into B is 100 and of B into A is 90, giving an initial net flux of 10. Assume that an allosteric effector increases the A $\rightarrow$ B rate by 20% to 120 and reciprocally decreases the B $\rightarrow$ A rate by 20% to 72. The new net flux is 48, and so a 20% change in the rates of the opposing reactions has led to a 380% increase in the net flux. In the example shown in Figure 16.32, this amplification is made possible by the rapid hydrolysis of ATP. It has been suggested that the flux down the glycolytic pathway may increase 1000-fold at the initiation of intense exercise. Because it seems unlikely that allosteric activation of enzymes alone could explain this increased flux, the existence of substrate cycles may partly account for the rapid rise in the rate of glycolysis.

The other potential biological role of substrate cycles is the *generation of heat produced by the hydrolysis of ATP*. A striking example is provided by bumblebees, which must maintain a thoracic temperature of about 30°C to fly. A bumblebee is able to maintain this high thoracic temperature and forage for food even when the ambient temperature is only 10°C because phosphofructokinase and fructose 1,6-bisphosphatase in its flight muscle are simultaneously highly active; the continuous hydrolysis of ATP generates heat. This bisphosphatase is not inhibited by AMP, which suggests that the enzyme is specially designed for the generation of heat. In contrast, the honeybee has almost no fructose 1,6-bisphosphatase activity in its flight muscle and consequently cannot fly when the ambient temperature is low.

16.4.2. Lactate and Alanine Formed by Contracting Muscle Are Used by Other Organs

Lactate produced by active skeletal muscle and erythrocytes is a source of energy for other organs. Erythrocytes lack mitochondria and can never oxidize glucose completely. In contracting skeletal muscle during vigorous exercise, the rate at which glycolysis produces pyruvate exceeds the rate at which the citric acid cycle oxidizes it. Under these conditions, moreover, the rate of formation of NADH by glycolysis is greater than the rate of its oxidation by aerobic metabolism. Continued glycolysis depends on the availability of NAD^+ for the oxidation of glyceraldehyde 3-phosphate. The accumulation of both NADH and pyruvate is reversed by lactate dehydrogenase, which oxidizes NADH to NAD^+ as it reduces pyruvate to lactate (Section 16.1.7). However, lactate is a dead end in metabolism. It must be converted back into pyruvate before it can be metabolized. The only purpose of the reduction of pyruvate to lactate is to regenerate NAD^+ so that glycolysis can proceed in active skeletal muscle and erythrocytes. *The formation of lactate buys time and shifts part of the metabolic burden from muscle to other organs*.

The plasma membrane of most cells contains carriers that render them highly permeable to lactate and pyruvate. Both substances diffuse out of active skeletal muscle into the blood and are carried to the liver. Much more lactate than pyruvate is transported out because the high $NADH/NAD^+$ ratio in contracting skeletal muscle favors the conversion of pyruvate into lactate. The lactate that enters the liver is oxidized to pyruvate, a reaction favored by the low $NADH/NAD^+$ ratio in the cytosol of liver cells. Pyruvate in the liver is converted into glucose by the gluconeogenic pathway. Glucose then enters the blood and is taken up by skeletal muscle. Thus, *the liver furnishes glucose to contracting skeletal muscle, which derives ATP from the glycolytic conversion of glucose into lactate. Contracting skeletal muscle supplies lactate to the liver, which uses it to synthesize glucose. These reactions constitute the Cori cycle* (Figure 16.33). Studies have shown that alanine, like lactate, is a major precursor of glucose. In muscle, alanine is formed from pyruvate by transamination (Section 24.2.2); the reverse reaction takes place in the liver. The interplay between glycolysis and gluconeogenesis is summarized in Figure 16.34, which shows how these two pathways help to meet the energy needs of different cell types.


Isozymic forms of lactate dehydrogenase in different tissues catalyze the interconversions of pyruvate and lactate. Lactate dehydrogenase is a tetramer of two kinds of 35-kd subunits encoded by similar genes: the H type predominates in the heart, and the homologous M type in skeletal muscle and the liver. These subunits associate to form five types of tetramers: H_4, H_3M_1, H_2M_2, H_1M_3, and M_4. The H_4 isozyme (type 1) has higher affinity for substrates than does the M_4 isozyme (type 5) and, unlike M_4, is allosterically inhibited by high levels of pyruvate. The other isozymes have intermediate properties, depending on the ratio of the two kinds of chains. H_4 is designed to oxidize lactate to

pyruvate, which is then utilized as a fuel by the heart through aerobic metabolism. Indeed, heart muscle never functions anaerobically. In contrast, M_4 is optimized to operate in the reverse direction, to convert pyruvate into lactate to allow glycolysis to proceed under anaerobic conditions. We see here an example of how gene duplication and divergence generate a series of homologous enzymes that foster metabolic cooperation between organs.

16.4.3. Glycolysis and Gluconeogenesis Are Evolutionarily Intertwined

The metabolism of glucose has ancient origins. Organisms living in the early biosphere depended on the anaerobic generation of energy until significant amounts of oxygen began to accumulate 2 billion years ago. The fact that glycolytic enzymes with similar properties do not have similar amino acid sequences also provides a clue to how the pathway originated. Although there are four kinases and two isomerases in the pathway, both sequence and structural comparisons do not suggest that these sets of enzymes are related to one another by divergent evolution. The absence of such similarities implies that glycolytic enzymes were derived independently rather than by gene duplication. The common dinucleotide-binding domain found in the dehydrogenases (Section 16.1.10) and the $\alpha\ \beta$ barrels are the only major recurring elements.

We can speculate on the relationship between glycolysis and gluconeogenesis if we think of glycolysis as consisting of two segments: the metabolism of hexoses (the upper segment) and the metabolism of trioses (the lower segment). The enzymes from the upper segment are different in some species and are missing entirely in some archaea, whereas enzymes from the lower segment are quite conserved. In fact, four enzymes of the lower segment are present in all species. *This lower part of the pathway is common to glycolysis and gluconeogenesis*. This common part of the two pathways may be the oldest part, constituting the core to which the other steps were added. The upper part would vary according to the sugars that were available to evolving organisms in particular niches. Interestingly, this core part of carbohydrate metabolism can generate triose precursors for ribose sugars, a component of RNA and a critical requirement for the RNA world. Thus, we are left with the unanswered question, Was the original core pathway used for energy conversion or biosynthesis?

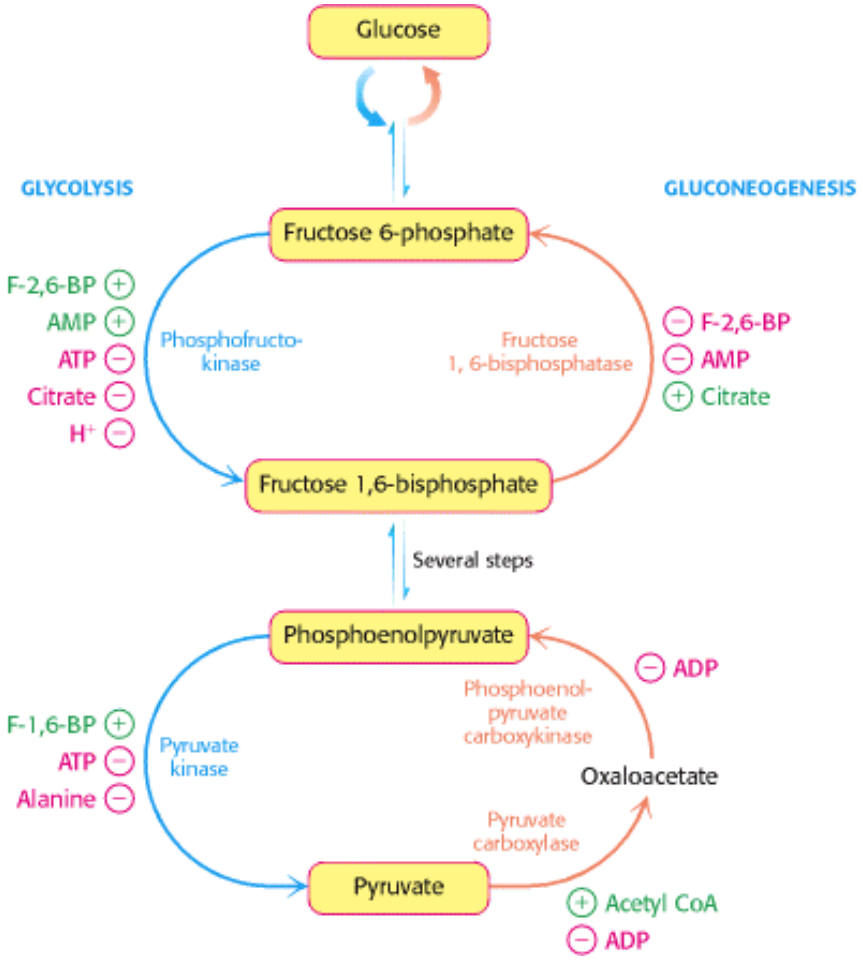


Figure 16.30. Reciprocal Regulation of Gluconeogenesis and Glycolysis in the Liver. The level of fructose 2,6-bisphosphate is high in the fed state and low in starvation. Another important control is the inhibition of pyruvate kinase by phosphorylation during starvation.

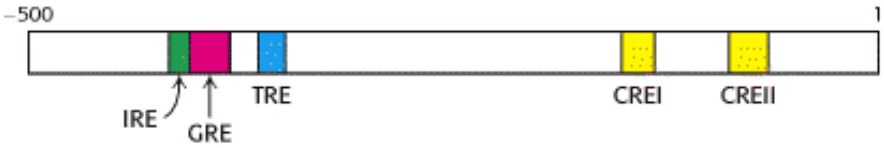


Figure 16.31. The Promoter of the Phosphoenolpyruvate Carboxykinase Gene. This promoter is approximately 500 bp in length and contains regulatory sequences (response elements) that mediate the action of several hormones. IRE, insulin response element; GRE, glucocorticoid response element; TRE, thyroid hormone response element; CREI and CREII, cAMP response elements. [After M. M. McGrane, J. S Jun, Y. M. Patel, and R. W. Hanson. *Trends Biochem. Sci.* 17(1992):40.]

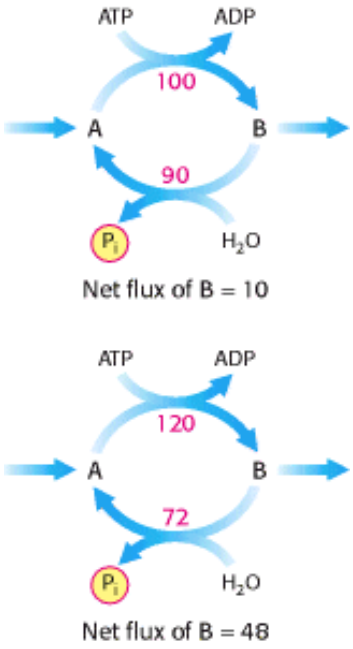


Figure 16.32. Substrate Cycle. This ATP-driven cycle operates at two different rates. A small change in the rates of the two opposing reactions results in a large change in the *net* flux of product B.

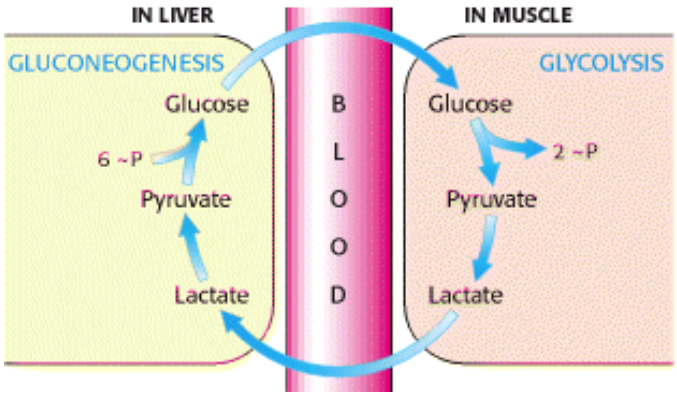


Figure 16.33. The Cori Cycle. Lactate formed by active muscle is converted into glucose by the liver. This cycle shifts part of the metabolic burden of active muscle to the liver.

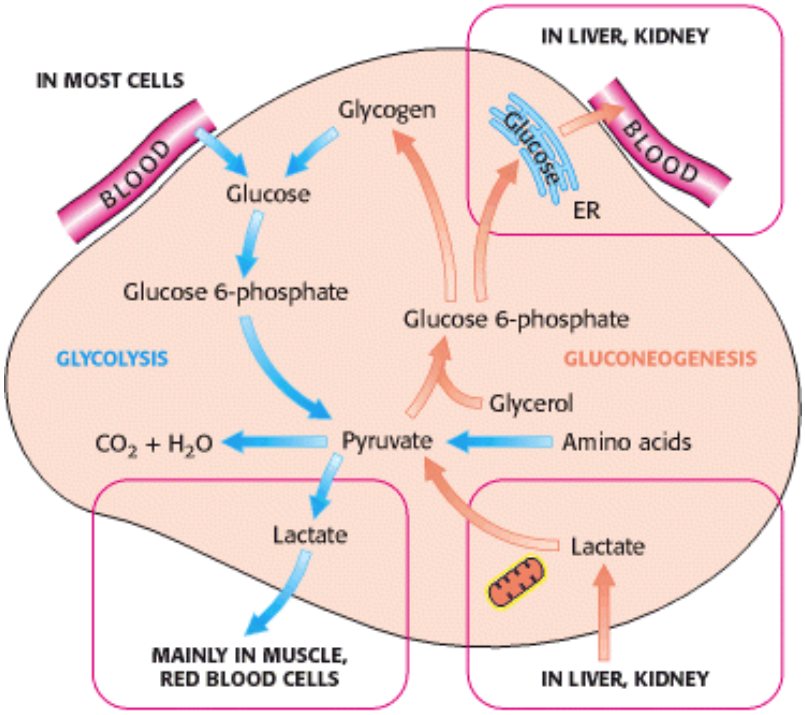


Figure 16.34. Cooperation between Glycolysis and Gluconeogenesis. Glycolysis and gluconeogenesis are coordinated, in a tissue-specific fashion, to ensure that the glucose-dependent energy needs of all cells are met.

Summary

Glycolysis Is an Energy-Conversion Pathway in Many Organisms

Glycolysis is the set of reactions that converts glucose into pyruvate. The 10 reactions of glycolysis take place in the cytosol. In the first stage, glucose is converted into fructose 1,6-bisphosphate by a phosphorylation, an isomerization, and a second phosphorylation reaction. Two molecules of ATP are consumed per molecule of glucose in these reactions, which are the prelude to the net synthesis of ATP. In the second stage, fructose 1,6-bisphosphate is cleaved by aldolase into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, which are readily interconvertible. In the third stage, ATP is generated. Glyceraldehyde 3-phosphate is oxidized and phosphorylated to form 1,3-bisphosphoglycerate, an acyl phosphate with a high phosphoryl-transfer potential. This molecule transfers a phosphoryl group to ADP to form ATP and 3-phosphoglycerate. A phosphoryl shift and a dehydration form phosphoenolpyruvate, a second intermediate with a high phosphoryltransfer potential. Another molecule of ATP is generated as phosphoenolpyruvate is converted into pyruvate. There is a net gain of two molecules of ATP in the formation of two molecules of pyruvate from one molecule of glucose.

The electron acceptor in the oxidation of glyceraldehyde 3-phosphate is NAD^+, which must be regenerated for glycolysis to continue. In aerobic organisms, the NADH formed in glycolysis transfers its electrons to O_2 through the electron-transport chain, which thereby regenerates NAD^+. Under anaerobic conditions and in some microorganisms, NAD^+ is regenerated by the reduction of pyruvate to lactate. In other microorganisms, NAD^+ is regenerated by the reduction of pyruvate to ethanol. These two processes are examples of fermentations.

The Glycolytic Pathway Is Tightly Controlled

The glycolytic pathway has a dual role: it degrades glucose to generate ATP, and it provides building blocks for the synthesis of cellular components. The rate of conversion of glucose into pyruvate is regulated to meet these two major cellular needs. Under physiologic conditions, the reactions of glycolysis are readily reversible except for the ones catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase. Phosphofructokinase, the most important control

element in glycolysis, is inhibited by high levels of ATP and citrate, and it is activated by AMP and fructose 2,6-bisphosphate. In the liver, this bisphosphate signals that glucose is abundant. Hence, phosphofructokinase is active when either energy or building blocks are needed. Hexokinase is inhibited by glucose 6-phosphate, which accumulates when phosphofructokinase is inactive. ATP and alanine allosterically inhibit pyruvate kinase, the other control site, and fructose 1,6-bisphosphate activates the enzyme. Consequently, pyruvate kinase is maximally active when the energy charge is low and glycolytic intermediates accumulate.

Glucose Can Be Synthesized from Noncarbohydrate Precursors

Gluconeogenesis is the synthesis of glucose from noncarbohydrate sources, such as lactate, amino acids, and glycerol. Several of the reactions that convert pyruvate into glucose are common to glycolysis. Gluconeogenesis, however, requires four new reactions to bypass the essential irreversibility of three reactions in glycolysis. In two of the new reactions, pyruvate is carboxylated in mitochondria to oxaloacetate, which in turn is decarboxylated and phosphorylated in the cytosol to phosphoenolpyruvate. Two high-energy phosphate bonds are consumed in these reactions, which are catalyzed by pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Pyruvate carboxylase contains a biotin prosthetic group. The other distinctive reactions of gluconeogenesis are the hydrolyses of fructose 1,6-bisphosphate and glucose 6-phosphate, which are catalyzed by specific phosphatases. The major raw materials for gluconeogenesis by the liver are lactate and alanine produced from pyruvate by active skeletal muscle. The formation of lactate during intense muscular activity buys time and shifts part of the metabolic burden from muscle to the liver.

Gluconeogenesis and Glycolysis Are Reciprocally Regulated

Gluconeogenesis and glycolysis are reciprocally regulated so that one pathway is relatively inactive while the other is highly active. Phosphofructokinase and fructose 1,6-bisphosphatase are key control points. Fructose 2,6-bisphosphate, an intracellular signal molecule present at higher levels when glucose is abundant, activates glycolysis and inhibits gluconeogenesis by regulating these enzymes. Pyruvate kinase and pyruvate carboxylase are regulated by other effectors so that both are not maximally active at the same time. Allosteric regulation and reversible phosphorylation, which are rapid, are complemented by transcriptional control, which takes place in hours or days.

Key Terms

glycolysis

lactic acid fermentation

alcoholic fermentation

gluconeogenesis

obligate anaerobe

facultative anaerobe

hexokinase

kinase

phosphofructokinase (PFK)

thioester intermediate

substrate-level phosphorylation

mutase

enol phosphate

pyruvate kinase

bifunctional enzyme

feedforward stimulation

committed step

pyruvate carboxylase

biotin

glucose 6-phosphatase

substrate cycle

Cori cycle

Problems

1. *Kitchen chemistry*. Sucrose is commonly used to preserve fruits. Why is glucose not suitable for preserving foods?

See answer

2. *Tracing carbon atoms I*. Glucose labeled with ^{14}C at C-1 is incubated with the glycolytic enzymes and necessary cofactors.

(a) What is the distribution of ^{14}C in the pyruvate that is formed? (Assume that the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate is very rapid compared with the subsequent step.)

(b) If the specific activity of the glucose substrate is 10 mCi mM^{-1}, what is the specific activity of the pyruvate that is formed?

See answer

3. *Lactic acid fermentation*. (a) Write a balanced equation for the conversion of glucose into lactate. (b) Calculate the standard free-energy change of this reaction by using the data given in Table 16.3 and the fact that $\Delta G^{\circ\prime}$ is -6 kcal for the following reaction:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$$

What is the free-energy change (ΔG, not $\Delta G^{\circ\prime}$) of this reaction when the concentrations of reactants are: glucose, 5 mM; lactate, 0.05 mM; ATP, 2 mM; ADP, 0.2 mM; and P_i, 1 mM?

See answer

4. *High potential*. What is the equilibrium ratio of phosphoenolpyruvate to pyruvate under standard conditions when [ATP]/[ADP] = 10?

See answer

5. *Hexose-triose equilibrium*. What are the equilibrium concentrations of fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate when 1 mM fructose 1,6-bisphosphate is incubated with aldolase under standard conditions?

See answer

6. *Double labeling*. 3-Phosphoglycerate labeled uniformly with ^{14}C is incubated with 1,3-BPG labeled with ^{32}P at C-1. What is the radioisotope distribution of the 2,3-BPG that is formed on addition of BPG mutase?

See answer

7. *An informative analog*. Xylose has the same structure as that of glucose except that it has a hydrogen atom at C-6 in place of a hydroxymethyl group. The rate of ATP hydrolysis by hexokinase is markedly enhanced by the addition of xylose. Why?

See answer

8. *Distinctive sugars*. The intravenous infusion of fructose into healthy volunteers leads to a two- to fivefold increase in the level of lactate in the blood, a far greater increase than that observed after the infusion of the same amount of glucose.

(a) Why is glycolysis more rapid after the infusion of fructose?

(b) Fructose has been used in place of glucose for intravenous feeding. Why is this use of fructose unwise?

See answer

9. *Metabolic mutants*. Predict the effect of each of the following mutations on the pace of glycolysis in liver cells:

(a) Loss of the allosteric site for ATP in phosphofructokinase.

(b) Loss of the binding site for citrate in phosphofructokinase.

(c) Loss of the phosphatase domain of the bifunctional enzyme that controls the level of fructose 2,6-bisphosphate.

(d) Loss of the binding site for fructose 1,6-bisphosphate in pyruvate kinase.

See answer

10. *Metabolic mutant*. What are the likely consequences of a genetic disorder rendering fructose 1,6-bisphosphatase in liver less sensitive to regulation by fructose 2,6-bisphosphate?

See answer

11. *Biotin snatcher*. Avidin, a 70-kd protein in egg white, has very high affinity for biotin. In fact, it is a highly specific inhibitor of biotin enzymes. Which of the following conversions would be blocked by the addition of avidin to a cell homogenate?

(a) Glucose $\rightarrow$ pyruvate

(b) Pyruvate $\rightarrow$ glucose

(c) Oxaloacetate $\rightarrow$ glucose

(d) Malate $\rightarrow$ oxaloacetate

(e) Pyruvate $\rightarrow$ oxaloacetate

(f) Glyceraldehyde 3-phosphate $\rightarrow$ fructose 1,6-bisphosphate

See answer

12. *Tracing carbon atoms II*. If cells synthesizing glucose from lactate are exposed to CO_2 labeled with ^{14}C, what will be the distribution of label in the newly synthesized glucose?

See answer

13. *Arsenate poisoning*. Arsenate (AsO_4^{3-}) closely resembles P_i in structure and reactivity. In the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase, arsenate can replace phosphate in attacking the energy-rich thioester intermediate. The product of this reaction, 1-arseno-3-phosphoglycerate, is unstable. It and other acyl arsenates are rapidly and spontaneously hydrolyzed. What is the effect of arsenate on energy generation in a cell?

See answer

14. *Reduce, reuse, recycle*. In the conversion of glucose into two molecules of lactate, the NADH generated earlier in the pathway is oxidized to NAD^+. Why is it not to the cells advantage to simply make more NAD^+ so that the regeneration would not be necessary? After all, the cell would save much energy because it would no longer need to synthesize lactic acid dehydrogenase.

See answer

15. *Adenylate kinase again*. Adenylate kinase, an enzyme discussed in great detail in Chapter 9, is responsible for interconverting the adenylate nucleotide pool:

$$ADP + ADP \underset{}{\overset{\text{Adenylate kinase}}{\rightleftharpoons}} ATP + AMP$$

The equilibrium constant for this reaction is close to 1, inasmuch as the number of phosphoanhydride bonds is the same on each side of the equation. Using the equation for the equilibrium constant for this reaction, show why changes in [AMP] are a more effective indicator of the adenylate pool than [ATP].

See answer

16. *Working at cross-purposes?* Gluconeogenesis takes place during intense exercise, which seems counterintuitive. Why would an organism synthesize glucose and at the same time use glucose to generate energy?

See answer

17. *Powering pathways*. Compare the stoichiometries of glycolysis and gluconeogenesis (Sections 16.1.8 and 16.3.6). Recall that the input of one ATP equivalent changes the equilibrium constant of a reaction by a factor of about 10^8 (Section 14.1.3). By what factor do the additional high phosphoryl-transfer potential compounds alter the equilibrium constant of gluconeogenesis?

See answer

Mechanism Problem

18. *Argument by analogy*. Propose a mechanism for the conversion of glucose 6-phosphate into fructose 6-phosphate by phosphoglucose isomerase based on the mechanism of triose phosphate isomerase.

See answer

Chapter Integration Problem

19. *Not just for energy*. People with galactosemia display central nervous system abnormalities even if galactose is eliminated from the diet. The precise reason for it is not known. Suggest a plausible explanation.

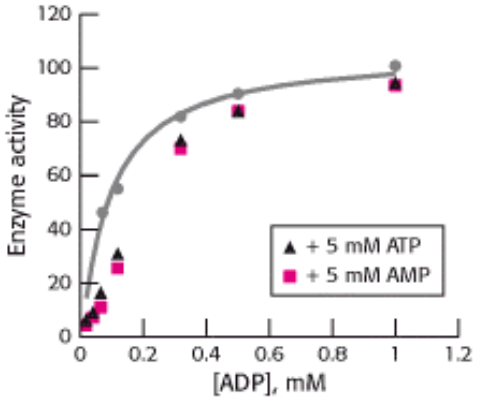
See answer

Data Interpretation Problem

20. *Now, that's unusual*. Phosphofructokinase has recently been isolated from the hyperthermophilic archaeon *Pyrococcus furiosus*. It was subjected to standard biochemical analysis to determine basic catalytic parameters. The processes under study were of the form:

$$\text{Fructose 6-phosphate} + (x - P_i) \longrightarrow \text{fructose 1,6-bisphosphate} + (x)$$

The assay measured the increase in fructose 1,6-bisphosphate. Selected results are shown in the adjoining graph.



[Data after: J. E. Tuininga et al. (1999) *J. Biol Chem.* 274:21023−21028.]

(a) How does the *P. furiosus* phosphofructokinase differ from the phosphofructokinase discussed in this chapter?

(b) What effects do AMP and ATP have on the reaction with ADP?

Media Problems

21. *No free lunch*. Suppose a microorganism is discovered to be lacking pyruvate kinase. Nevertheless, the organism does metabolize glucose to pyruvate. Surprisingly, it does so with a net gain of four molecules of ATP (or ATP equivalents) per molecule of metabolized glucose. Looking at the **Conceptual Insights** module on the energetics of glucose metabolism, suggest a biochemical pathway for metabolism of glucose in this organism. Why might evolution have favored the normal glycolytic pathway rather than this alternative?

22. *Oxidation in the absence of oxygen*. The energetics of glycolysis section of the **Conceptual Insights** module shows that there is a large free energy drop upon oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (reaction 6). In the presence of oxygen, some to this energy is ultimately converted into ATP production. However, no such conversion happens under anaerobic conditions. Explain why.

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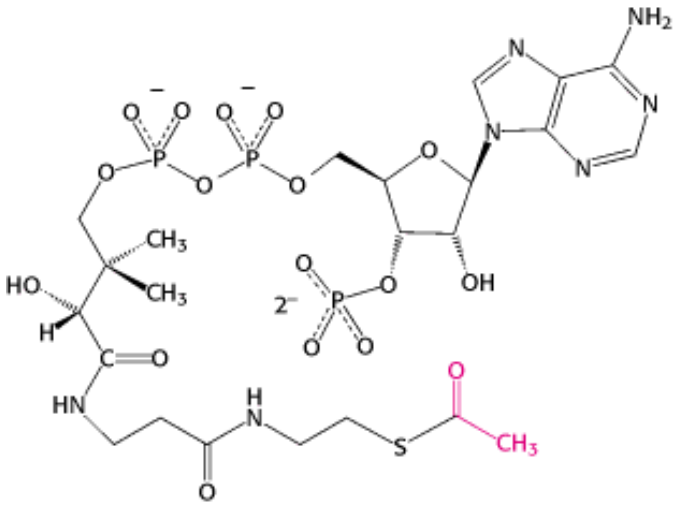
17. The Citric Acid Cycle

From Chapter 16, we know that glucose can be metabolized to pyruvate anaerobically to synthesize ATP through the glycolytic pathway. Glycolysis, however, harvests but a fraction of the ATP available from glucose. We now begin an exploration of the aerobic processing of glucose, which is the source of most of the ATP generated in metabolism. The aerobic processing of glucose starts with the complete oxidation of glucose derivatives to carbon dioxide. This oxidation takes place in the *citric acid cycle*, a series of reactions also known as the *tricarboxylic acid (TCA) cycle* or the *Krebs cycle*. The citric acid cycle is the *final common pathway for the oxidation of fuel molecules* —amino acids, fatty acids, and carbohydrates. Most fuel molecules enter the cycle as acetyl coenzyme A.



Acetyl coenzyme A (Acetyl CoA)

Under aerobic conditions, the pyruvate generated from glucose is oxidatively decarboxylated to form acetyl CoA. In eukaryotes, the reactions of the citric acid cycle take place inside mitochondria, in contrast with those of glycolysis, which take place in the cytosol (Figure 17.1).

17.0.1. An Overview of the Citric Acid Cycle

The citric acid cycle is the central metabolic hub of the cell. It is the gateway to the aerobic metabolism of any molecule that can be transformed into an acetyl group or dicarboxylic acid. The cycle is also an important source of precursors, not only for the storage forms of fuels, but also for the building blocks of many other molecules such as amino acids, nucleotide bases, cholesterol, and porphyrin (the organic component of heme).

What is the function of the citric acid cycle in transforming fuel molecules into ATP? Recall that fuel molecules are carbon compounds that are capable of being oxidized—of losing electrons (Chapter 14). The citric acid cycle includes a series of oxidation-reduction reactions that result in the oxidation of an acetyl group to two molecules of carbon dioxide.

The overall pattern of the citric acid cycle is shown in Figure 17.2. A four- carbon compound (oxaloacetate) condenses with a two-carbon acetyl unit to yield a six-carbon tricarboxylic acid (citrate). An isomer of citrate is then oxidatively decarboxylated. The resulting five-carbon compound (α-ketoglutarate) also is oxidatively decarboxylated to yield a four-carbon compound (succinate). Oxaloacetate is then regenerated from succinate. Two carbon atoms enter the cycle as an acetyl unit and two carbon atoms leave the cycle in the form of two molecules of carbon dioxide. Three hydride ions (hence, six electrons) are transferred to three molecules of nicotinamide adenine dinucleotide (NAD^+), whereas one pair of hydrogen atoms (hence, two electrons) is transferred to one molecule of flavin adenine dinucleotide (FAD). *The function of the citric acid cycle is the harvesting of high-energy electrons from carbon fuels.* Note that the citric acid cycle itself neither generates a large amount of ATP nor includes oxygen as a reactant (Figure 17.3). Instead, the citric acid cycle removes electrons from acetyl CoA and uses these electrons to form NADH and $FADH_2$. In *oxidative phosphorylation* (Chapter 18), electrons released in the reoxidation of NADH and $FADH_2$ flow through a series of membrane proteins (referred to as the *electron-transport chain*) to generate a proton gradient across the membrane. These protons then flow through ATP synthase to generate ATP from ADP and inorganic phosphate. Oxygen is required for the citric acid cycle indirectly inasmuch as it is the electron acceptor at the end of the electron-transport chain, necessary to regenerate NAD^+ and FAD.

The citric acid cycle, in conjunction with oxidative phosphorylation, provides the vast majority of energy used by aerobic cells—in human beings, greater than 95%. It is highly efficient because a limited number of molecules can generate large amounts of NADH and $FADH_2$. Note in Figure 17.2 that the four-carbon molecule, oxaloacetate, that initiates the first step in the citric acid cycle is regenerated at the end of one passage through the cycle. The oxaloacetate acts catalytically: it participates in the oxidation of the acetyl group but is itself regenerated. Thus, one molecule of

oxaloacetate is capable of participating in the oxidation of many acetyl molecules.

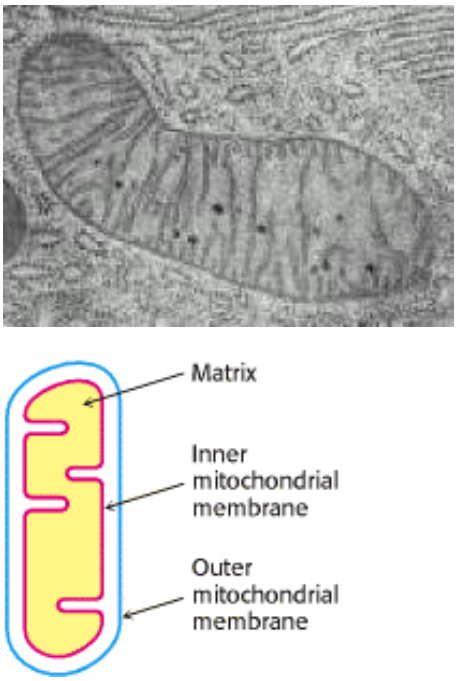


Figure 17.1. Mitochondrion. The double membrane of the mitochondrion is evident in this electron micrograph. The numerous invaginations of the inner mitochondrial membrane are called cristae. The oxidative decarboxylation of pyruvate and the sequence of reactions in the citric acid cycle take place within the matrix. [(Left) Omikron/Photo Researchers.]

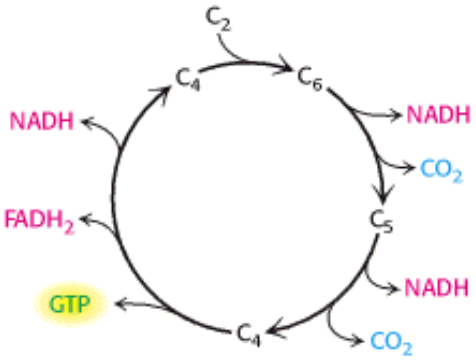


Figure 17.2. Overview of the Citric Acid Cycle. The citric acid cycle oxidizes two-carbon units, producing two molecules of CO_2, one molecule of GTP, and high-energy electrons in the form of NADH and $FADH_2$.

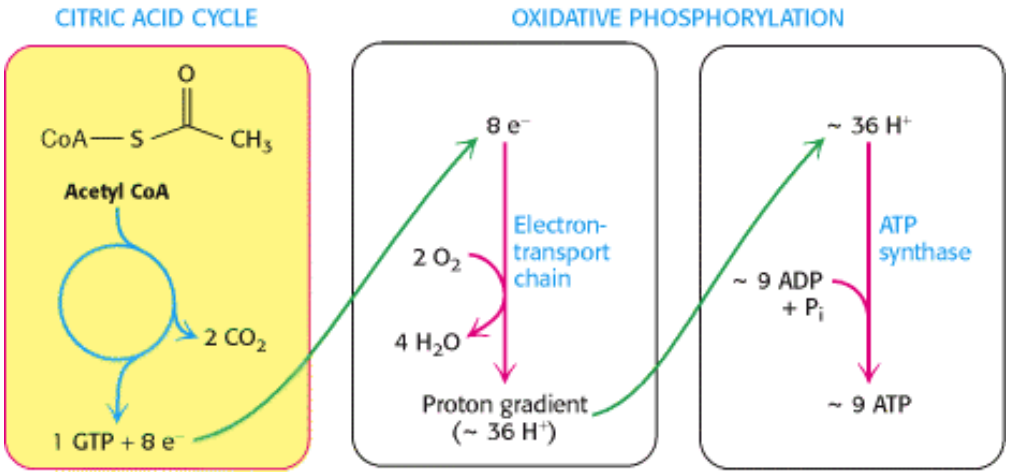
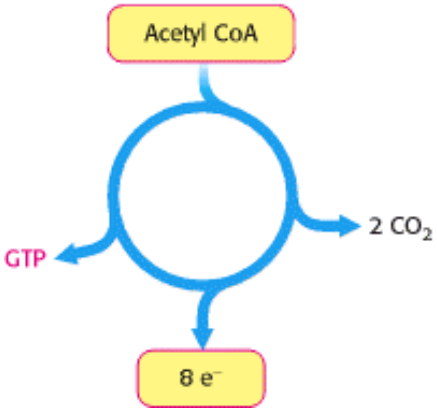


Figure 17.3. Cellular Respiration. The citric acid cycle constitutes the first stage in cellular respiration, the removal of high-energy electrons from carbon fuels (left). These electrons reduce O_2 to generate a proton gradient (middle), which is used to synthesize ATP (right). The reduction of O_2 and the synthesis of ATP constitute oxidative phosphorylation.



Roundabouts, or traffic circles, function as hubs to facilitate traffic flow. The citric acid cycle is the biochemical hub of the cell, oxidizing carbon fuels, usually in the form of acetyl CoA, as well as serving as a source of precursors for biosynthesis. [(Above) Chris Warren/International Stock.]

17.1. The Citric Acid Cycle Oxidizes Two-Carbon Units

Acetyl CoA is the fuel for the citric acid cycle. This important molecule is formed from the breakdown of glycogen (the storage form of glucose), fats, and many amino acids. Indeed, as we will see in Chapter 22, fats contain strings of reduced two-carbon units that are first oxidized to acetyl CoA and then completely oxidized to CO_2 by the citric acid cycle.

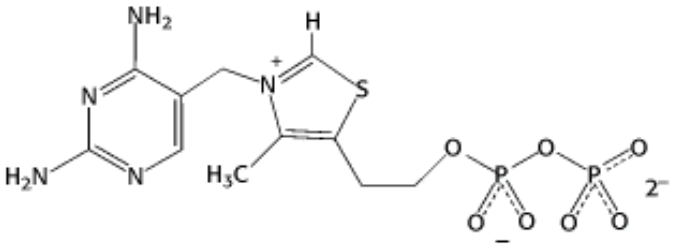
17.1.1. The Formation of Acetyl Coenzyme A from Pyruvate

The formation of acetyl CoA from carbohydrates is less direct than from fat. Recall that carbohydrates, most notably glucose, are processed by glycolysis into pyruvate (Chapter 16). Under anaerobic conditions, the pyruvate is converted into lactic acid or ethanol, depending on the organism. Under aerobic conditions, the pyruvate is transported into mitochondria in exchange for OH^- by the pyruvate carrier, an antiporter (Section 13.4). In the mitochondrial matrix, pyruvate is oxidatively decarboxylated by the *pyruvate dehydrogenase complex* to form acetyl CoA.

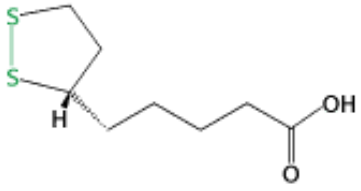
$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{acetyl CoA} + \text{CO}_2 + \text{NADH}$$

This irreversible reaction is the link between glycolysis and the citric acid cycle. (Figure 17.4) Note that, in the preparation of the glucose derivative pyruvate for the citric acid cycle, an oxidative decarboxylation takes place and high-transfer-potential electrons in the form of NADH are captured. Thus, the pyruvate dehydrogenase reaction has many of the key features of the reactions of the citric acid cycle itself.

The pyruvate dehydrogenase complex is a large, highly integrated complex of three kinds of enzymes (Table 17.1). Pyruvate dehydrogenase is a member of a family of homologous complexes that includes the citric acid cycle enzyme α-ketoglutarate dehydrogenase (Section 17.1.6), a branched-chain α-ketoacid dehydrogenase, and acetoin dehydrogenase, found in certain prokaryotes. These complexes are giant, with molecular masses ranging from 4 to 10 million daltons (Figure 17.5). As we will see, their elaborate structures allow groups to travel from one active site to another, connected by tethers to the core of the structure. The mechanism of the pyruvate dehydrogenase reaction is wonderfully complex, more so than is suggested by its relatively simple stoichiometry. The reaction requires the participation of the three enzymes of the pyruvate dehydrogenase complex, each composed of several polypeptide chains, and five coenzymes: *thiamine pyrophosphate (TPP)*, *lipoic acid*, and *FAD* serve as catalytic cofactors, and CoA and NAD^+ are stoichiometric cofactors.



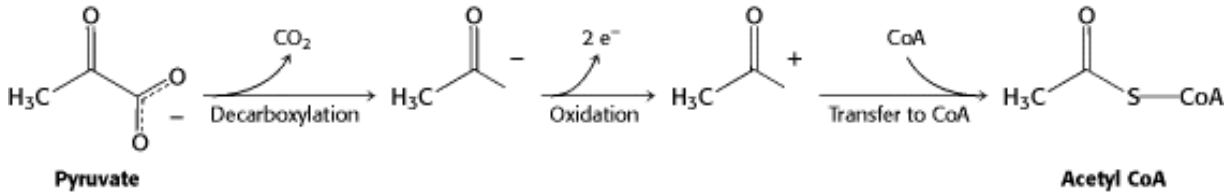
Thiamine pyrophosphate (TPP)



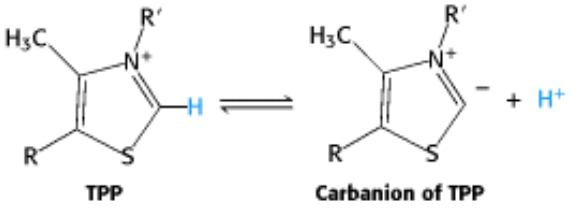
Lipoic acid

At least two additional enzymes regulate the activity of the complex.

The conversion of pyruvate into acetyl CoA consists of three steps: decarboxylation, oxidation, and transfer of the resultant acetyl group to CoA.

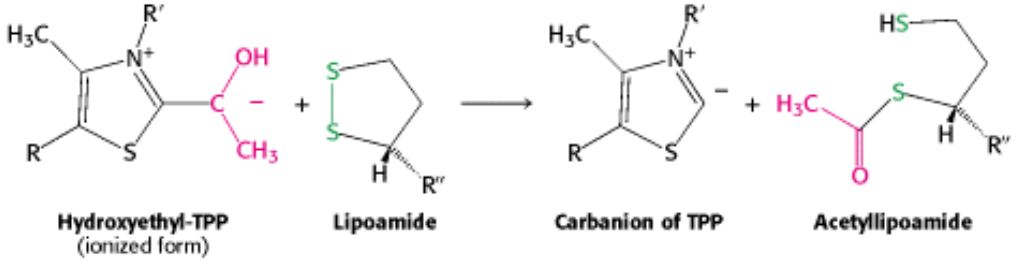


These steps must be coupled to preserve the free energy derived from the decarboxylation step to drive the formation of NADH and acetyl CoA. First, pyruvate combines with TPP and is then decarboxylated (Figure 17.6). This reaction is catalyzed by the *pyruvate dehydrogenase component* (E_1) of the multienzyme complex. A key feature of TPP, the prosthetic group of the pyruvate dehydrogenase component, is that the carbon atom between the nitrogen and sulfur atoms in the thiazole ring is much more acidic than most =CH- groups, with a pK_a value near 10. This center ionizes to form a *carbanion*, which readily adds to the carbonyl group of pyruvate.

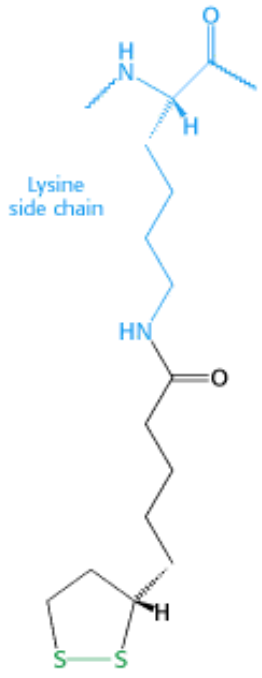


This addition is followed by the decarboxylation of pyruvate. The positively charged ring of TPP acts as an electron sink that stabilizes the negative charge that is transferred to the ring as part of the decarboxylation. Protonation yields hydroxyethyl-TPP.

Second, the hydroxyethyl group attached to TPP is *oxidized* to form an acetyl group and concomitantly *transferred* to lipoamide, a derivative of lipoic acid that is linked to the side chain of a lysine residue by an amide linkage.

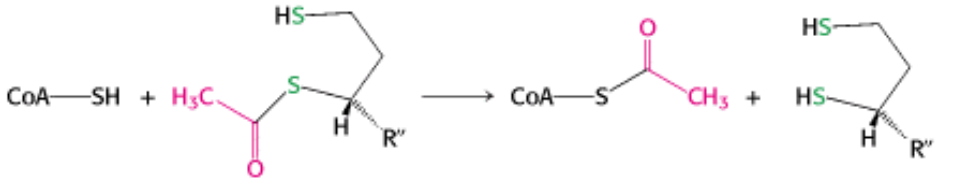


The oxidant in this reaction is the disulfide group of lipoamide, which is reduced to its disulfhydryl form. This reaction, also catalyzed by the pyruvate dehydrogenase component E_1, yields *acetyllipoamide.*



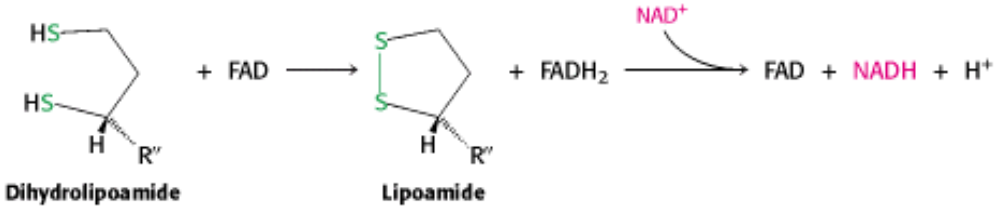
Lipoamide

Third, *the acetyl group is transferred from acetyllipoamide to CoA to form acetyl CoA.*



Dihydrolipoyl transacetylase (E_2) catalyzes this reaction. The energy-rich thioester bond is preserved as the acetyl group is transferred to CoA. Recall that CoA serves as a carrier of many activated acyl groups, of which acetyl is the simplest (Section 14.3.1). Acetyl CoA, the fuel for the citric acid cycle, has now been generated from pyruvate.

The pyruvate dehydrogenase complex cannot complete another catalytic cycle until the dihydrolipoamide is oxidized to lipoamide. In a fourth step, *the oxidized form of lipoamide is regenerated by dihydrolipoyl dehydrogenase* (E_3). Two electrons are transferred to an FAD prosthetic group of the enzyme and then to NAD^+.



This electron transfer to FAD is unusual, because the common role for FAD is to receive electrons from NADH. The electron transfer potential of FAD is altered by its association with the enzyme and enables it to transfer electrons to NAD^+. Proteins tightly associated with FAD or flavin mononucleotide (FMN) are called *flavoproteins.*

17.1.2. Flexible Linkages Allow Lipoamide to Move Between Different Active Sites

Although the structure of an intact member of the pyruvate dehydrogenase complex family has not yet been determined in atomic detail, the structures of all of the component enzymes are now known, albeit from different complexes and species. Thus, it is now possible to construct an atomic model of the complex to understand its activity (Figure 17.7).

The core of the complex is formed by E_2. Acetyltransferase consists of eight catalytic trimers assembled to form a hollow cube. Each of the three subunits forming a trimer has three major domains (Figure 17.8). At the amino terminus is a small domain that contains a bound lipoamide cofactor attached to a lysine residue. This domain is homologous to biotin-binding domains such as that of pyruvate carboxylase (see Figure 16.26). The lipoamide domain is followed by a small domain that interacts with E_3 within the complex. A larger transacetylase domain completes an E_2 subunit. E_1 is an $\alpha_2\beta_2$ tetramer, and E_3 is a $\alpha\beta$ dimer. Twenty-four copies of E_1 and 12 copies of E_3 surround the E_2 core. How do the three distinct active sites work in concert (Figure 17.9)?

1. Pyruvate is decarboxylated at the active site of E_1, forming the substituted TPP intermediate, and CO_2 leaves as the first product. This active site lies within the E_1 complex, connected to the enzyme surface by a 20-Å-long hydrophobic channel.

2. E_2 inserts the lipoyl-lysine arm of the lipoamide domain into the channel in E_1.

3. E_1 catalyzes the transfer of the acetyl group to the lipoamide. The acetylated lipoyl-lysine arm then leaves E_1 and enters the E_2 cube through 30 Å windows on the sides of the cube to visit the active site of E_2, located deep in the cube at the subunit interface.

4. The acetyl moiety is then transferred to CoA, and the second product, acetyl CoA, leaves the cube. The reduced lipoyl-lysine arm then swings to the active site of the E_3 flavoprotein.

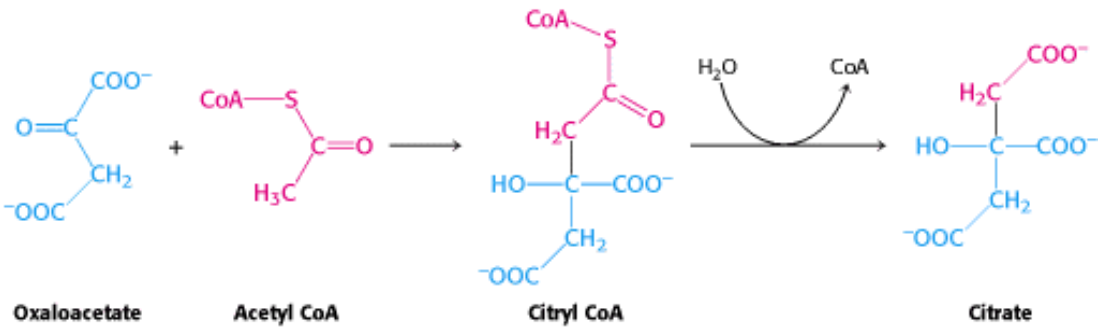
5. At the E_3 active site, the lipoamide acid is oxidized by coenzyme FAD.

6. The final product, NADH, is produced with the reoxidation of $FADH_2$, and the reactivated lipoamide is ready to begin another reaction cycle.

The structural integration of three kinds of enzymes makes the coordinated catalysis of a complex reaction possible. The proximity of one enzyme to another *increases the overall reaction rate* and *minimizes side reactions.* All the intermediates in the oxidative decarboxylation of pyruvate are tightly bound to the complex and are readily transferred because of the ability of the lipoyl-lysine arm of E_2 to call on each active site in turn.

17.1.3. Citrate Synthase Forms Citrate from Oxaloacetate and Acetyl Coenzyme A

The citric acid cycle begins with the condensation of a four-carbon unit, oxaloacetate, and a two-carbon unit, the acetyl group of acetyl CoA. Oxaloacetate reacts with acetyl CoA and H_2O to yield citrate and CoA.



This reaction, which is an aldol condensation followed by a hydrolysis, is catalyzed by *citrate synthase.* Oxaloacetate first condenses with acetyl CoA to form *citryl CoA,* which is then hydrolyzed to citrate and CoA. The hydrolysis of citryl CoA, a high-energy thioester intermediate, drives the overall reaction far in the direction of the synthesis of citrate. In essence, the hydrolysis of the thioester powers the synthesis of a new molecule from two precursors. Because this reaction initiates the cycle, it is very important that side reactions be minimized. Let us briefly consider the how citrate synthase prevents wasteful processes such as the hydrolysis of acetyl CoA.

Synthase-

An enzyme catalyzing a synthetic reaction in which two units are joined without the direct participation of ATP (or another nucleoside triphosphate).

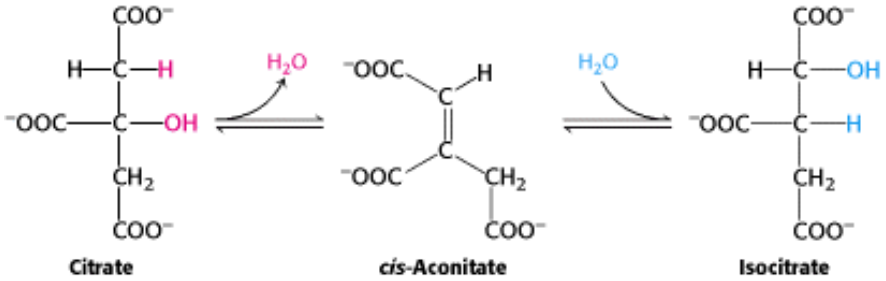
Mammalian citrate synthase is a dimer of identical 49-kd subunits. Each active site is located in a cleft between the large and small domains of a subunit, adjacent to the subunit interface. The results of x-ray crystallographic studies of citrate synthase and its complexes with several substrates and inhibitors revealed that the enzyme undergoes large conformational changes in the course of catalysis. Citrate synthase exhibits sequential, ordered kinetics: oxaloacetate binds first, followed by acetyl CoA. The reason for the ordered binding is that *oxaloacetate induces a major structural rearrangement leading to the creation of a binding site for acetyl CoA.* The open form of the enzyme observed in the absence of ligands is converted into a closed form by the binding of oxaloacetate (Figure 17.10). In each subunit, the small domain rotates 19 degrees relative to the large domain. *Movements as large as 15 Å are produced by the rotation of* α *helices elicited by quite small shifts of side chains around bound oxaloacetate.* This conformational transition is reminiscent of the cleft closure in hexokinase induced by the binding of glucose (Section 16.1.1).

Citrate synthase catalyzes the condensation reaction by bringing the substrates into close proximity, orienting them, and polarizing certain bonds. Two histidine residues and an aspartate residue are important players (Figure 17.11). One of the histidine residues (His 274) donates a proton to the carbonyl oxygen of acetyl CoA to promote the removal of a methyl proton by Asp 375. Oxaloacetate is activated by the transfer of a proton from His 320 to its carbonyl carbon atom. The concomitant attack of the enol of acetyl CoA on the carbonyl carbon of oxaloacetate results in the formation of a carbon-carbon bond. The newly formed citryl CoA induces additional structural changes in the enzyme. The active site becomes completely enclosed. His 274 participates again as a proton donor to hydrolyze the thioester. Coenzyme A leaves the enzyme, followed by citrate, and the enzyme returns to the initial open conformation.

We can now understand how the wasteful hydrolysis of acetyl CoA is prevented. Citrate synthase is well suited to hydrolyze *citryl* CoA but not *acetyl* CoA. How is this discrimination accomplished? First, acetyl CoA does not bind to the enzyme until oxaloacetate is bound and ready for condensation. Second, the catalytic residues crucial for hydrolysis of the thioester linkage are not appropriately positioned *until citryl CoA is formed.* As with hexokinase (Section 16.1.1) and triose phosphate isomerase (Section 16.1.4), *induced fit prevents an undesirable side reaction.*

17.1.4. Citrate Is Isomerized into Isocitrate

The tertiary hydroxyl group is not properly located in the citrate molecule for the oxidative decarboxylations that follow. Thus, citrate is isomerized into isocitrate to enable the six-carbon unit to undergo oxidative decarboxylation. The isomerization of citrate is accomplished by a *dehydration* step followed by a *hydration* step. The result is an interchange of a hydrogen atom and a hydroxyl group. The enzyme catalyzing both steps is called *aconitase* because cis-*aconitate* is an intermediate.



Aconitase is an *iron-sulfur protein*, or *nonheme iron protein.* It contains four iron atoms that are not incorporated as part of a heme group. The four iron atoms are complexed to four inorganic sulfides and three cysteine sulfur atoms, leaving one iron atom available to bind citrate and then isocitrate through their carboxylate and hydroxyl groups (Figure 17.12). This iron center, in conjunction with other groups on the enzyme, facilitates the dehydration and rehydration reactions. We will consider the role of these iron-sulfur clusters in the electron-transfer reactions of oxidative phosphorylation subsequently (Section 18.3.1).

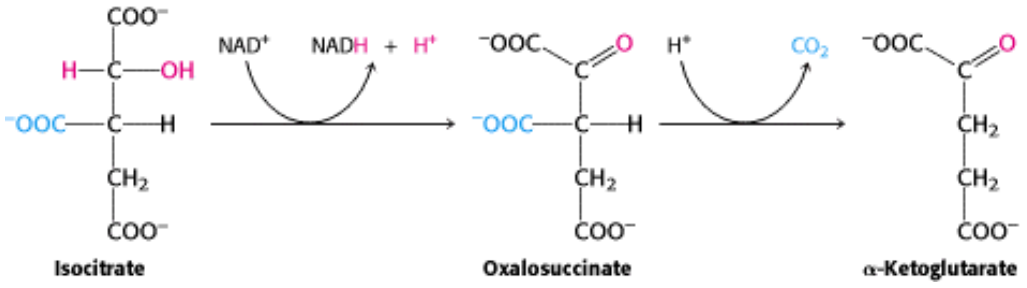
The iron-sulfur cluster in aconitase is somewhat unstable, so one or more iron atoms dissociate under conditions of low iron availability in the cell. Remarkably, this sensitivity to iron level was exploited in the evolution of a mechanism for regulating gene expression in response to iron levels, as will be discussed in Chapter 31.

17.1.5. Isocitrate Is Oxidized and Decarboxylated to α-Ketoglutarate

We come now to the first of four oxidation-reduction reactions in the citric acid cycle. The oxidative decarboxylation of isocitrate is catalyzed by *isocitrate dehydrogenase.*

$$\text{Isocitrate} + \text{NAD}^+ \longrightarrow \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADH}$$

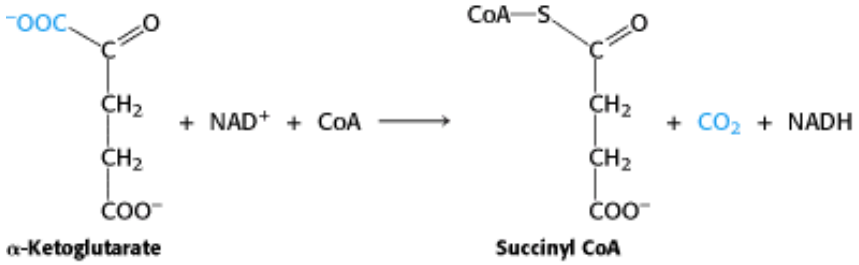
The intermediate in this reaction is oxalosuccinate, an unstable β-ketoacid. While bound to the enzyme, it loses CO_2 to form α-ketoglutarate.



The rate of formation of α-ketoglutarate is important in determining the overall rate of the cycle, as will be discussed in Section 17.2.2. This oxidation generates the first high-transfer-potential electron carrier NADH in the cycle.

17.1.6. Succinyl Coenzyme A Is Formed by the Oxidative Decarboxylation of α-Ketoglutarate

The conversion of isocitrate into α-ketoglutarate is followed by a second oxidative decarboxylation reaction, the formation of succinyl CoA from α-ketoglutarate.



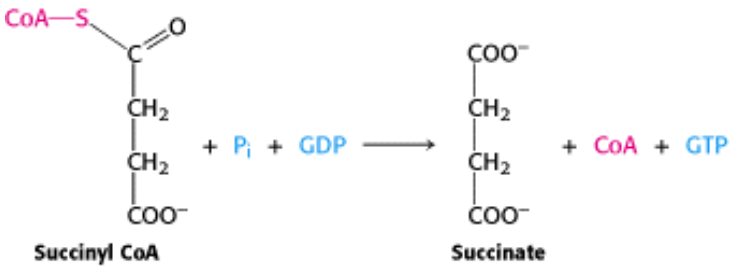
The oxidative decarboxylation of α-ketoglutarate closely resembles that of pyruvate, also an α-ketoacid.

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{acetyl CoA} + \text{CO}_2 + \text{NADH}$$

Both reactions include the decarboxylation of an α-ketoacid and the subsequent formation of a high-transfer-potential thioester linkage with CoA. The complex that catalyzes the oxidative decarboxylation of α-ketoglutarate is homologous to the pyruvate dehydrogenase complex, and the reaction mechanism is entirely analogous. The α-*ketoglutarate dehydrogenase* component (E_2) and transsuccinylase (E_1) are different from but homologous to the corresponding enzymes in the pyruvate dehydrogenase complex, whereas the dihydrolipoyl dehydrogenase components (E_3) of the two complexes are identical.

17.1.7. A High Phosphoryl-Transfer Potential Compound Is Generated from Succinyl Coenzyme A

Succinyl CoA is an energy-rich thioester compound. The $\Delta G^{\circ\prime}$ for the hydrolysis of succinyl CoA is about -8 kcal mol^{-1} (-33.5 kJ mol^{-1}), which is comparable to that of ATP (-7.3 kcal mol^{-1}, or -30.5 kJ mol^{-1}). In the ci-trate synthase reaction, the cleavage of the thioester bond powers the synthesis of the six-carbon citrate from the four-carbon oxaloacetate and the two-carbon fragment. *The cleavage of the thioester bond of succinyl CoA is coupled to the phosphorylation of a purine nucleoside diphosphate, usually GDP*. This reaction is catalyzed by *succinyl CoA synthetase* (succinate thiokinase). This enzyme is an $\alpha_2\beta_2$ heterodimer; the functional unit is one $\alpha\beta$ pair. The mechanism is a clear example of energy transformations: energy inherent in the thioester molecule is transformed into phosphoryl-group transfer potential (Figure 17.13). The first step is the displacement of coenzyme A by orthophosphate, which generates another energy-rich compound, succinyl phosphate. A histidine residue of the α subunit removes the phosphoryl group with the concomitant generation of succinate and phosphohistidine. The phosphohistidine residue then swings over to a bound nucleoside diphosphate and the phosphoryl group is transferred to form the nucleoside triphosphate. The participation of high-energy compounds in all the steps is attested to by the fact that the reaction is readily reversible: $\Delta G^{\circ\prime}$ = -0.8 kcal mol^{-1} (-3.4 kJ mol^{-1}). This is the only step in the citric acid cycle that directly yields a compound with high phosphoryl transfer potential through a substrate-level phosphorylation. Some mammalian succinyl CoA synthetases are specific for GDP and others for ADP. The *E. coli* enzyme uses either GDP or ADP as the phosphoryl-group acceptor. We have already seen that GTP is an important component of signal-transduction systems (Chapter 15). Alternatively, its γ-phosphoryl group can be readily transferred to ADP to form ATP, in a reaction catalyzed by *nucleoside diphosphokinase.*

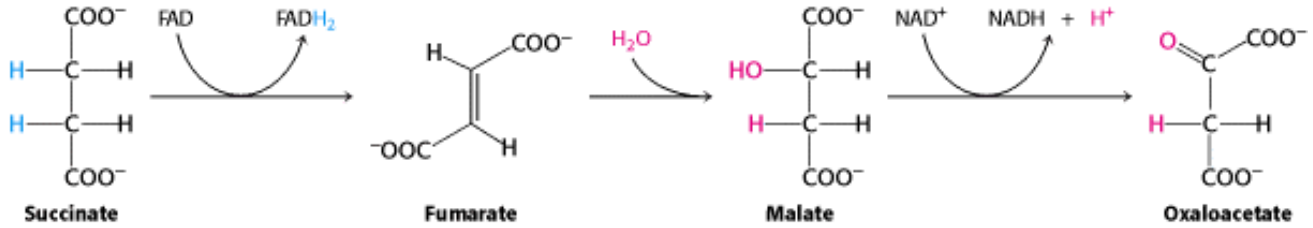


$$\text{GTP} + \text{ADP} \rightleftharpoons \text{GDP} + \text{ATP}$$

The mechanism of succinyl CoA synthetase reveals that a phosphoryl group is transferred first to succinyl CoA bound in the α subunit and then to a nucleoside diphosphate bound in the β subunit. Examination of the three-dimensional structure of succinyl CoA synthetase shows that each subunit comprises two domains (Figure 17.14). The carboxyl-terminal domains of the two subunits are similar to one another, whereas the amino-terminal domains have different structures, each characteristic of its role in the mechanism. The amino-terminal domain of the α subunit forms a Rossmann fold (Section 16.1.10), which binds the ADP component of succinyl CoA, whereas the amino-terminal domain of the β subunit is an ATP-grasp domain, a nucleotide-activating domain found in many enzymes, especially those catalyzing purine biosynthesis (Section 16.3.2 and Chapter 25). Succinyl CoA synthetase has evolved by adopting these domains and harnessing them to allow the capture of the energy associated with succinyl CoA cleavage to drive the generation of a nucleoside triphosphate.

17.1.8. Oxaloacetate Is Regenerated by the Oxidation of Succinate

Reactions of four-carbon compounds constitute the final stage of the citric acid cycle: the regeneration of oxaloacetate.



The reactions constitute a metabolic motif that we will see again in fatty acid synthesis and degradation as well as in the degradation of some amino acids (see Figure 14.17). A methylene group (CH_2) is converted into a carbonyl group (C = O) in three steps: an oxidation, a hydration, and a second oxidation reaction. Not only is oxaloacetate thereby regenerated for another round of the cycle, but also more energy is extracted in the form of $FADH_2$ and NADH.

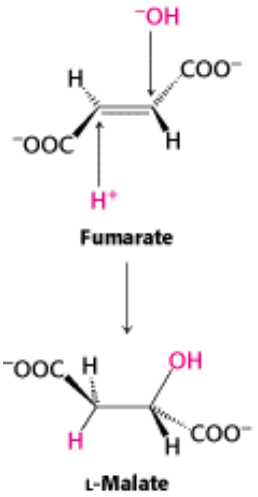
Succinate is oxidized to fumarate by *succinate dehydrogenase.* The hydrogen acceptor is FAD rather than NAD^+, which is used in the other three oxidation reactions in the cycle. In succinate dehydrogenase, the isoalloxazine ring of FAD is covalently attached to a histidine side chain of the enzyme (denoted E-FAD).

$$\text{E-FAD} + \text{succinate} \rightleftharpoons \text{E-FADH}_2 + \text{fumarate}$$

FAD is the hydrogen acceptor in this reaction because the free-energy change is insufficient to reduce NAD^+. FAD is nearly always the electron acceptor in oxidations that remove two hydrogen *atoms* from a substrate.

Succinate dehydrogenase, like aconitase, is an iron-sulfur protein. Indeed, succinate dehydrogenase contains three different kinds of iron-sulfur clusters, 2Fe-2S (two iron atoms bonded to two inorganic sulfides), 3Fe-4S, and 4Fe-4S. Succinate dehydrogenase—which consists of two subunits, one 70 kd and the other 27 kd—differs from other enzymes in the citric acid cycle in being embedded in the inner mitochondrial membrane. In fact, *succinate dehydrogenase is directly associated with the electron-transport chain, the link between the citric acid cycle and ATP formation.* $FADH_2$ produced by the oxidation of succinate does not dissociate from the enzyme, in contrast with NADH produced in other oxidation-reduction reactions. Rather, two electrons are transferred from $FADH_2$ directly to iron-sulfur clusters of the enzyme. The ultimate acceptor of these electrons is molecular oxygen, as we shall see in Chapter 18.

The next step is the hydration of fumarate to form l-malate. *Fumarase* catalyzes a stereospecific trans addition of a hydrogen atom and a hydroxyl group. The hydroxyl group adds to only one side of the double bond of fumarate; hence, only the l isomer of malate is formed.



Finally, malate is oxidized to form oxaloacetate. This reaction is catalyzed by *malate dehydrogenase*, and NAD^+ is again the hydrogen acceptor.

$$\text{Malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+$$

Note that the standard free energy for this reaction, unlike that for the other steps in the citric acid cycle, is significantly positive. The oxidation of malate is driven by the utilization of the products—oxaloacetate by citrate synthase and NADH by the electron-transport chain.

17.1.9. Stoichiometry of the Citric Acid Cycle

The net reaction of the citric acid cycle is:

$$\text{Acetyl CoA} + 3\,NAD^+ + FAD + GDP + P_i + 2\,H_2O \longrightarrow 2\,CO_2 + 3\,NADH + FADH_2 + GTP + 2\,H^+ + CoA$$

Let us recapitulate the reactions that give this stoichiometry (Figure 17.15 and Table 17.2):

1. Two carbon atoms enter the cycle in the condensation of an acetyl unit (from acetyl CoA) with oxaloacetate. Two carbon atoms leave the cycle in the form of CO_2 in the successive decarboxylations catalyzed by isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. Interestingly, the results of isotope-labeling studies revealed that the two carbon atoms that enter each cycle are not the ones that leave.

2. Four pairs of hydrogen atoms leave the cycle in four oxidation reactions. Two molecules of NAD^+ are reduced in the oxidative decarboxylations of isocitrate and α -ketoglutarate, one molecule of FAD is reduced in the oxidation of succinate, and one molecule of NAD^+ is reduced in the oxidation of malate.

3. One compound with high phosphoryl transfer potential, usually GTP, is generated from the cleavage of the thioester linkage in succinyl CoA.

4. Two molecules of water are consumed: one in the synthesis of citrate by the hydrolysis of citryl CoA and the other in the hydration of fumarate.

Recall also that NADH is generated in the formation of acetyl CoA from pyruvate by the pyruvate dehydrogenase reaction.

The efficiency of the citric acid cycle may be enhanced by the arrangement of the constituent enzymes. Evidence is accumulating that the enzymes are physically associated with one another to facilitate substrate channeling between active sites. The word *metabolon* has been suggested as the name for such multienzyme complexes.

As will be discussed in Chapter 18, the electron-transport chain oxidizes the NADH and $FADH_2$ formed in the citric acid cycle. The transfer of electrons from these carriers to O_2, the ultimate electron acceptor, leads to the generation of a proton gradient across the inner mitochondrial membrane. This proton-motive force then powers the generation of ATP; the net stoichiometry is about 2.5 ATP per NADH, and 1.5 ATP per $FADH_2$. Consequently, 9 high-transfer-potential phosphoryl groups are generated when the electron-transport chain oxidizes 3 molecules of NADH and 1 molecule of $FADH_2$, and 1 high-transfer-potential phosphoryl group per acetyl unit is directly formed in the citric acid cycle. Thus, 1 acetate unit generates approximately 10 molecules of ATP. In dramatic contrast, only 2 molecules of ATP are generated per molecule of glucose (which generates 2 molecules of acetyl CoA) by anaerobic glycolysis.

Recall that molecular oxygen does not participate directly in the citric acid cycle. However, the cycle operates only under aerobic conditions because NAD^+ and FAD can be regenerated in the mitochondrion only by the transfer of electrons to molecular oxygen. *Glycolysis has both an aerobic and an anaerobic mode, whereas the citric acid cycle is strictly aerobic.* Glycolysis can proceed under anaerobic conditions because NAD^+ is regenerated in the conversion of pyruvate into lactate.

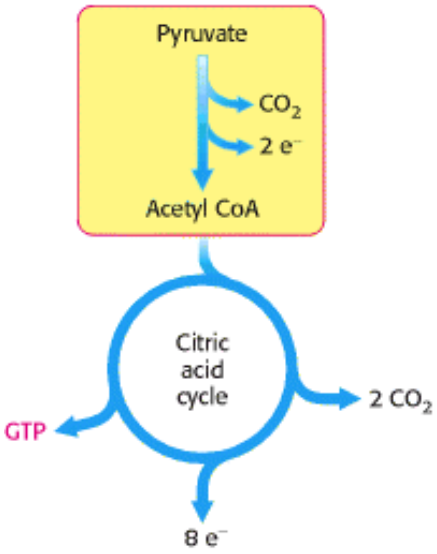


Figure 17.4. The Link between Glycolysis and the Citric Acid Cycle. Pyruvate produced by glycolysis is converted into acetyl CoA, the fuel of the citric acid cycle.

Table 17.1. Pyruvate dehydrogenase complex of *E. coli*

Enzyme	Abbreviation	Number of chains	Prosthetic group	Reaction catalyzed
Pyruvate dehydrogenase component	E_1	24	TPP	Oxidative decarboxylation of pyruvate
Dihydrolipoyl transacetylase	E_2	24	Lipoamide	Transfer of the acetyl group to CoA

Dihydrolipoyl dehydrogenase	E_3	12	FAD	Regeneration of the oxidized form of lipoamide

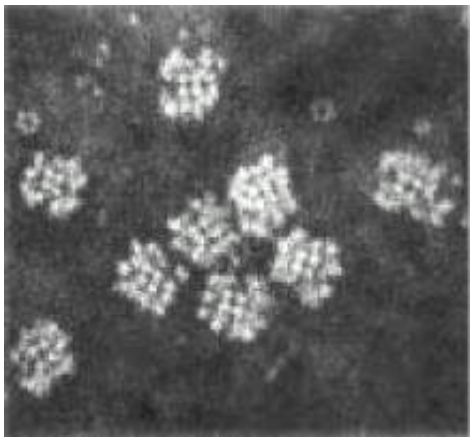


Figure 17.5. Electron Micrograph of the Pyruvate Dehydrogenase Complex From *E. coli.* [Courtesy of Dr. Lester Reed.]

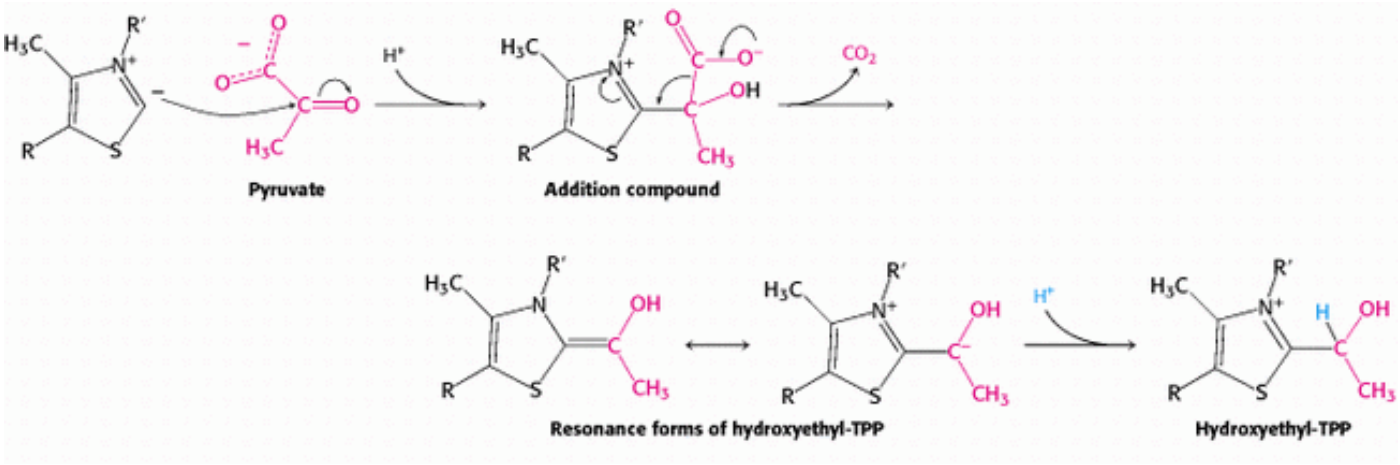


Figure 17.6. Mechanism of the Decarboxylation Reaction of E_1 , THe Pyruvate Dehydrogenase Component of the Pyruvate Dehydrogenese Complex.

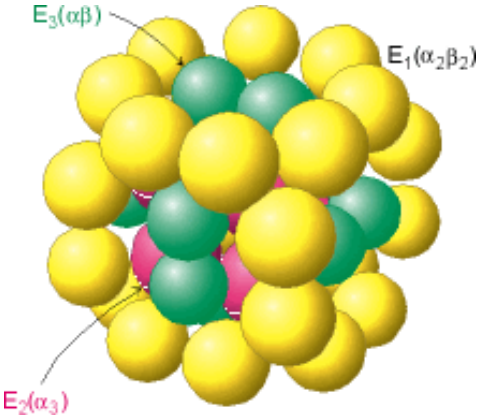


Figure 17.7. Schematic Representation of the Pyruvate Dehydrogenase Complex. The transacetylase core (E_2) is shown in red, the pyruvate dehydrogenase component (E_1) in yellow, and the dihydrolipoyl dehydrogenase (E_3) in green.

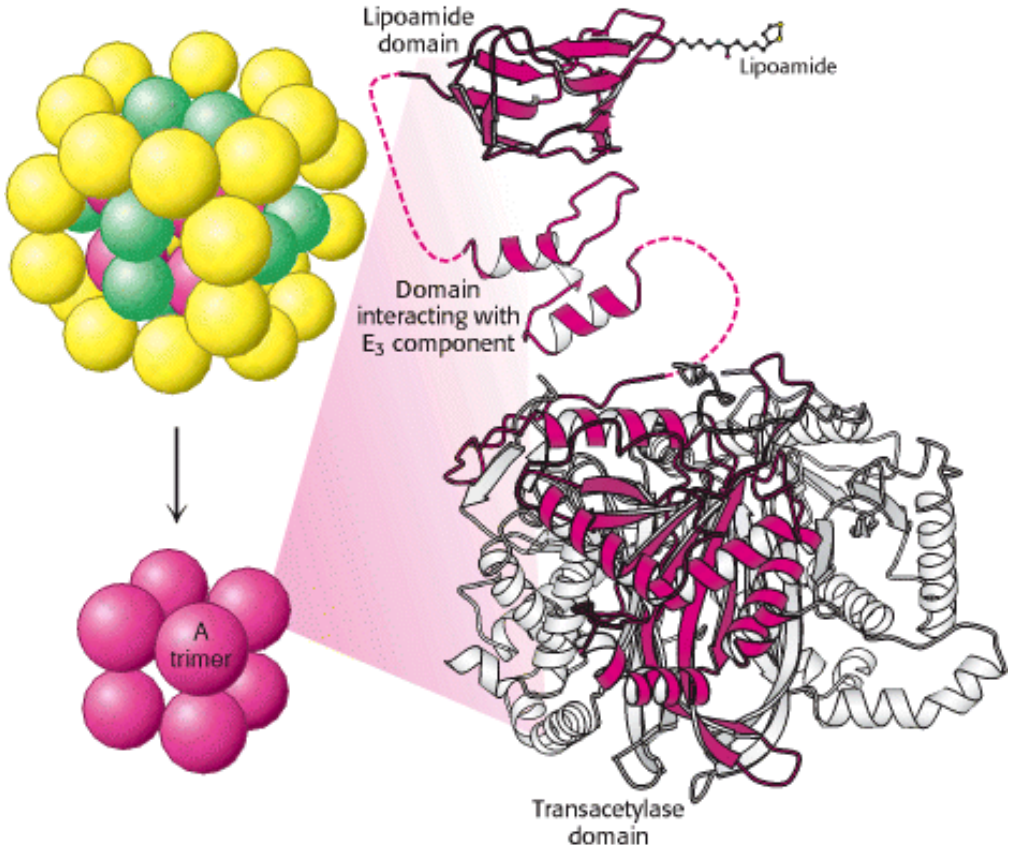


Figure 17.8. Structure of the Transacetylase (E_2) core. Each red ball represents a trimer of three E_2 subunits. Each subunit consists of three domains: a lipoamide-binding domain, a small domain for interaction with E_3, and a large transacetylase catalytic domain. All three subunits of the transacetylase domain are shown in the ribbon representation, with one depicted in red.

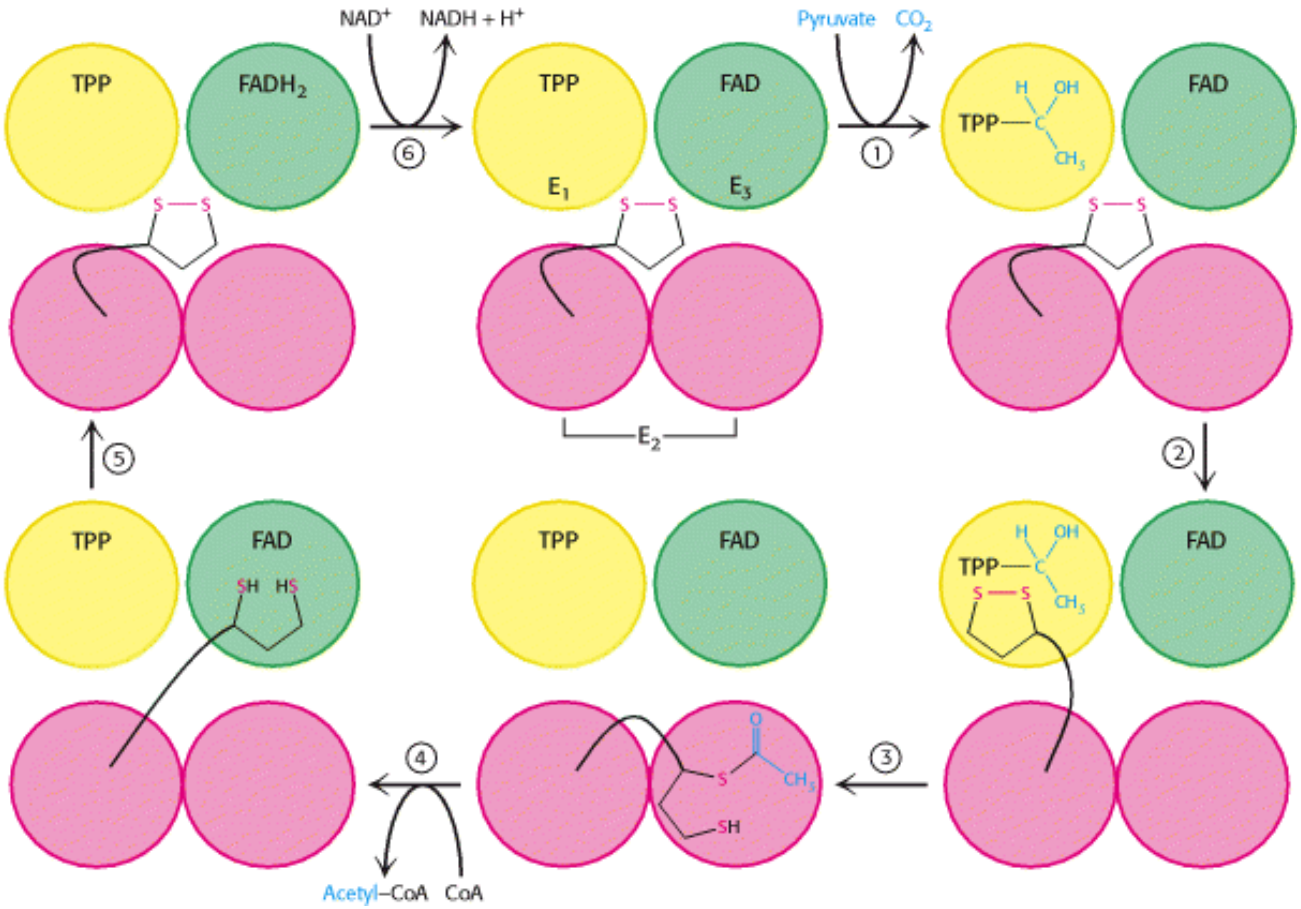


Figure 17.9. Reactions of the Pyruvate Dehydrogenase Complex. At the top (center), the enzyme (represented by a yellow, a blue, and two red spheres) is unmodified and ready for a catalytic cycle. (1) Pyruvate is decarboxylated to form the hydroxyethyl TPP. (2) The dihydrolipoyl arm of E_2 moves into the active site of E_1. (3) E_1 catalyzes the transfer of the two-carbon group to the dihydrolipoyl group to form the acetyl-lipoyl complex. (4) E_2 catalyzes the transfer of the acetyl moiety to CoA to form the product acetyl CoA. The disulfhydryl lipoyl arm then swings to the active site of E_3. E_3 catalyzes (5) the reduction of the lipoic acid and (6) the transfer of the protons and electrons to NAD^+ to complete the reaction cycle.

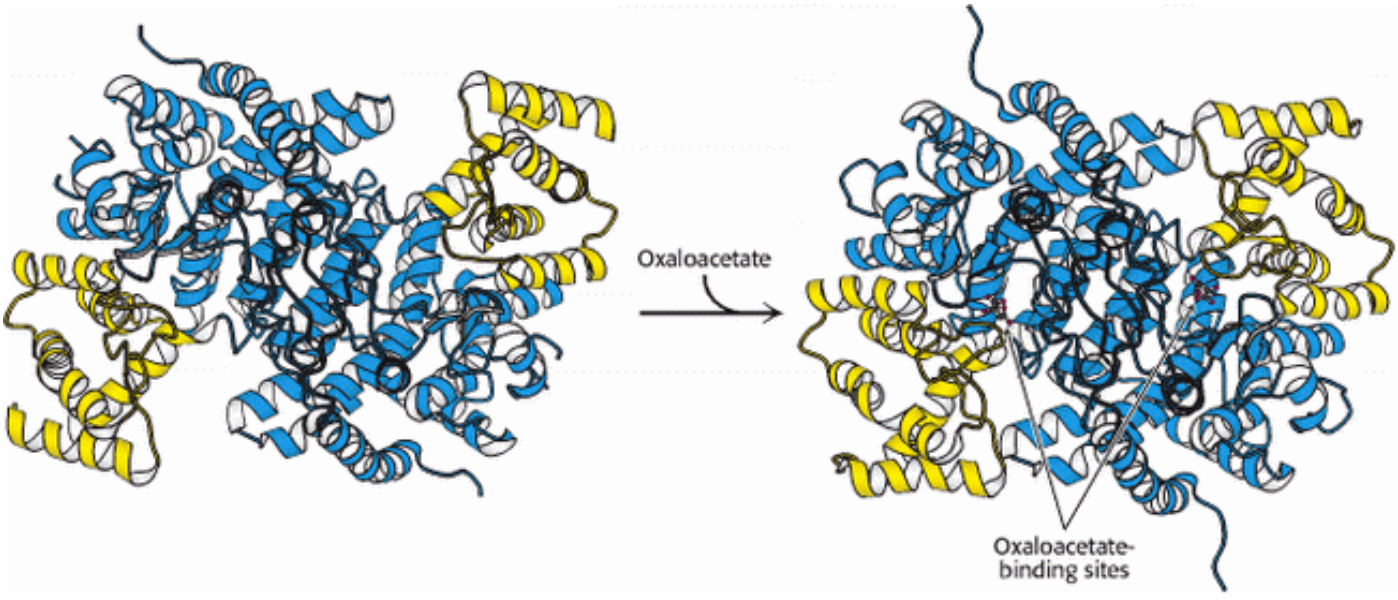


Figure 17.10. Conformational Changes in Citrate Synthase on Binding Oxaloacetate. The small domain of each

subunit of the homodimer is shown in yellow; the large domain is shown in blue. (Left) Open form of enzyme alone. (Right) Closed form of the liganded enzyme.

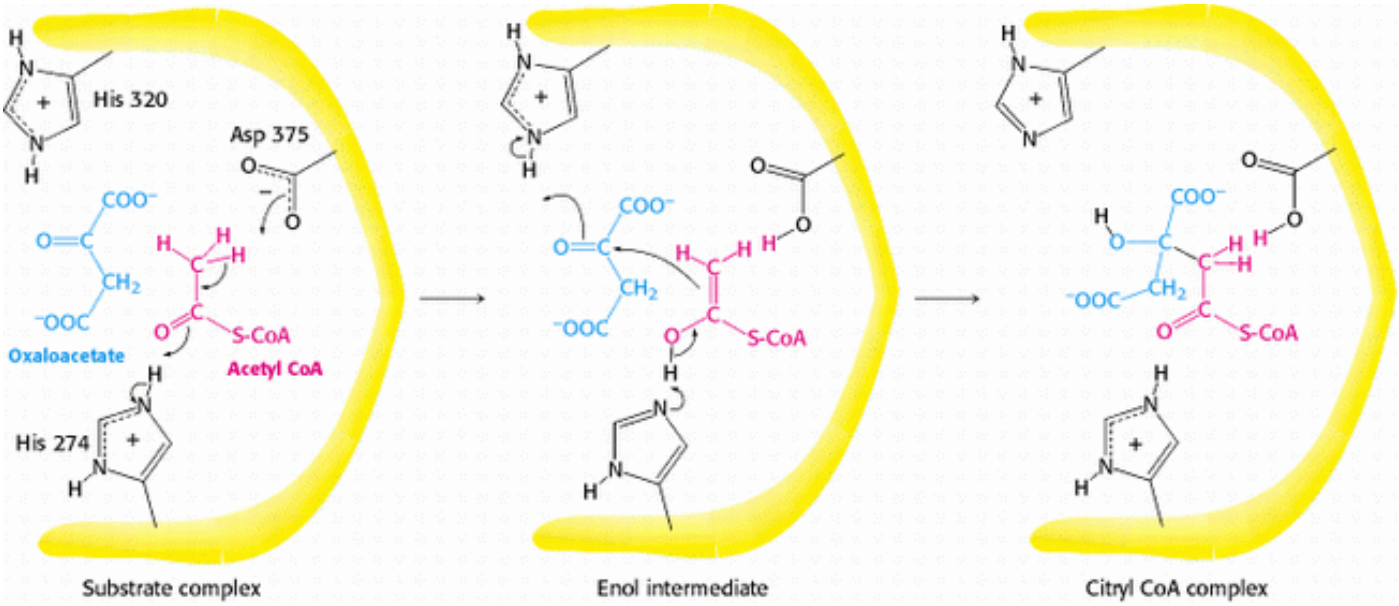


Figure 17.11. Mechanism of Synthesis of Citryl CoA by Citrate Synthase. The condensation of oxaloacetate and acetyl CoA proceeds through an enol intermediate. The subsequent hydrolysis of citryl CoA yields citrate and CoA.

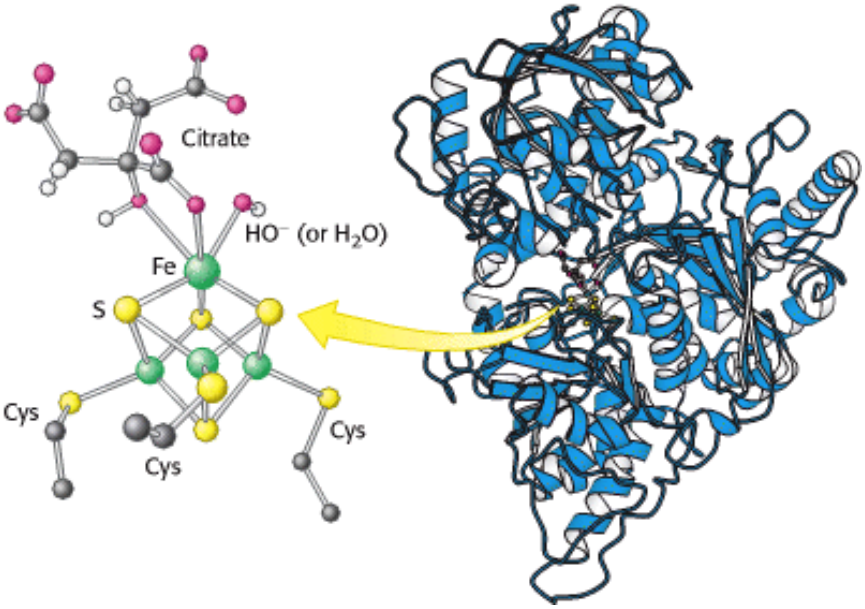


Figure 17.12. Binding of Citrate to the Iron-Sulfur Complex of Aconitase. A 4Fe-4S iron-sulfur cluster is a component of the active site of aconitase. One of the iron atoms of the cluster binds to the carboxylate and hydroxyl groups of citrate.

Figure 17.13. Reaction Mechanism of Succinyl CoA Synthetase. The formation of GTP at the expense of succinyl CoA is an example of substrate-level phosphorylation. The reaction proceeds through a phosphorylated enzyme intermediate.

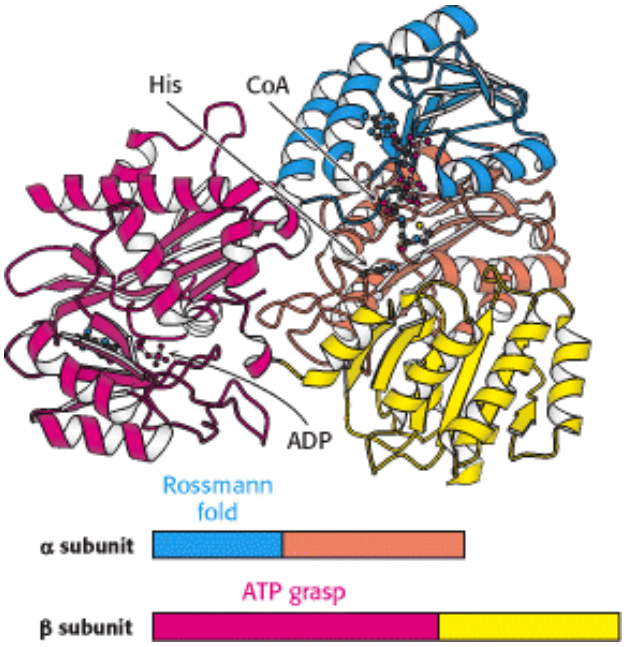


Figure 17.14. Structure of Succinyl CoA Synthetase. The enzyme is composed of two subunits. The α subunit contains a Rossmann fold that binds the ADP component of CoA, and the β subunit contains a nucleotide-activating region called the ATP-grasp domain. The ATP-grasp domain is shown here binding a molecule of ADP. The histidine residue picks up the phosphoryl group from near the CoA and swings over to transfer it to the nucleotide bound in the ATP-grasp domain.

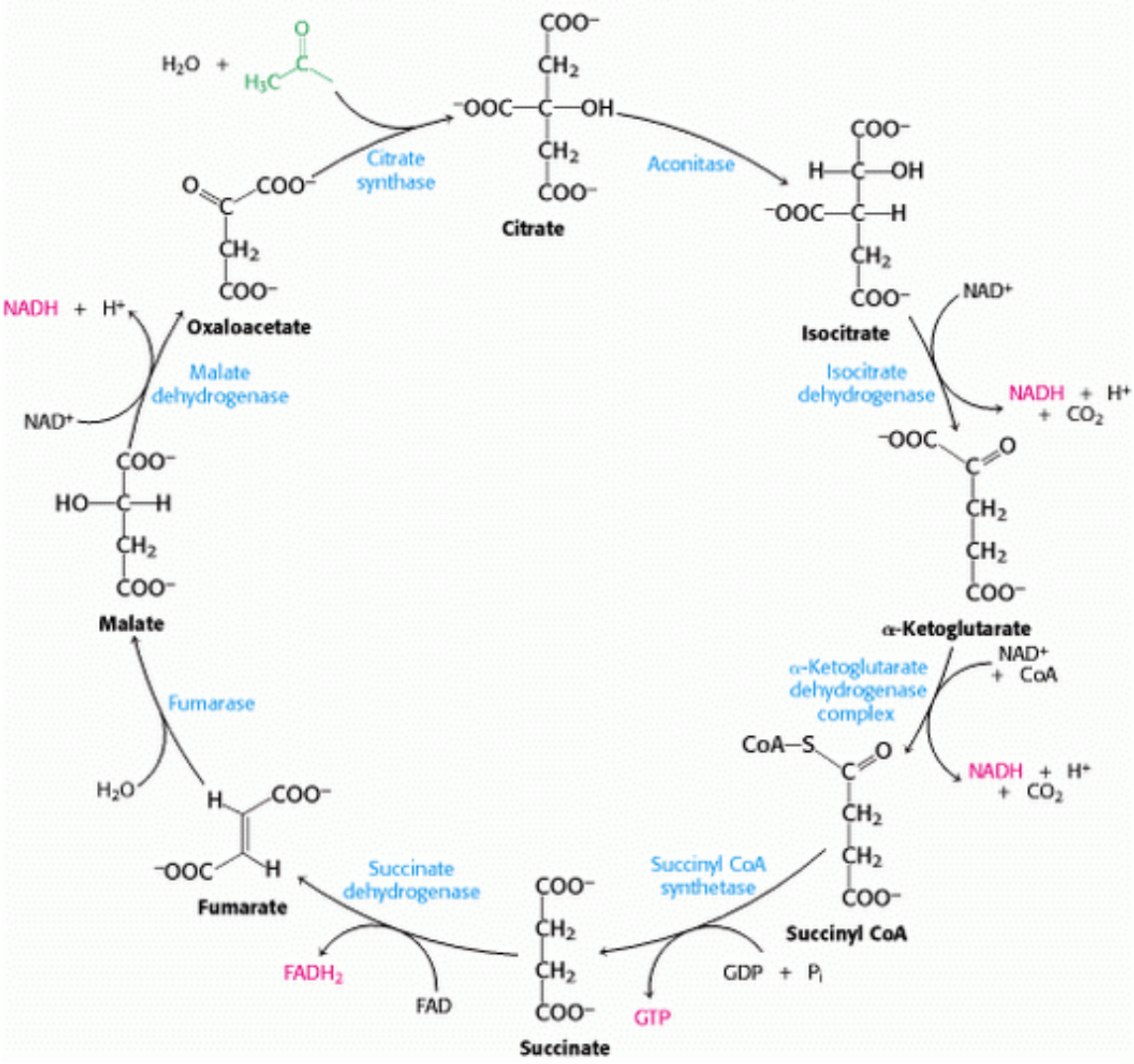


Figure 17.15. The Citric Acid Cycle.

Table 17.2. Citric acid cycle

Step	Reaction	Enzyme	Prosthetic group	Type*	DG° kcal mol^{-1}	DG° kJ mol^{-1}
1	Acetyl CoA + oxaloacetate + $H_2O \rightarrow$ citrate + CoA + H^+	Citrate synthase		a	-7.5	-31.4
2a	Citrate $\rightleftharpoons$ *cis*-aconitate + H_2O	Aconitase	Fe-S	b	+2.0	+8.4
2b	*cis*-Aconitate+ $H_2O \rightleftharpoons$ isocitrate	Aconitase	Fe-S	c	-0.5	-2.1
3	Isocitrate + $NAD^+ \rightleftharpoons$ α-ketoglutarate + CO_2 + NADH	Isocitrate dehydrogenase		d + e	-2.0	-8.4

4	α-Ketoglutarate + NAD^+ + CoA ⇌ succinyl CoA + CO_2 + NADH	α-Ketoglutarate dehydrogenase complex	Lipoic acid, FAD, TPP	d + e	-7.2	-30.1
5	Succinyl CoA + P_i + GDP ⇌ succinate + GTP + CoA	Succinyl CoA synthetase		f	-0.8	-3.3
6	Succinate + FAD (enzyme-bound) ⇌ fumarate + $FADH_2$ (enzyme-bound)	Succinate dehydrogenase	FAD, Fe-S	e	˜0	0
7	Fumarate + H_2O ⇌ l-malate	Furmarase		c	-0.9	-3.8
8	l-Malate + NAD^+ ⇌ oxaloacetate + NADH + H^+	Malate dehydrogenase		e	+7.1	+29.7

* Reaction type: (a) condensation; (b) dehydration; (c) hydration; (d) decarboxylation; (e) oxidation; (f) substrate-level phosphorylation.

17.2. Entry to the Citric Acid Cycle and Metabolism Through It Are Controlled

The citric acid cycle is the final common pathway for the aerobic oxidation of fuel molecules. Moreover, as we will see shortly (Section 17.3) and repeatedly elsewhere in our study of biochemistry, the cycle is an important source of building blocks for a host of important biomolecules. As befits its role as the metabolic hub of the cell, entry into the cycle and the rate of the cycle itself are controlled at several stages.

17.2.1. The Pyruvate Dehydrogenase Complex Is Regulated Allosterically and by Reversible Phosphorylation

As we saw earlier, glucose can be formed from pyruvate (Section 16.3). *However, the formation of acetyl CoA from pyruvate is an irreversible step in animals and thus they are unable to convert acetyl CoA back into glucose.* The oxidative decarboxylation of pyruvate to acetyl CoA commits the carbon atoms of glucose to two principal fates: oxidation to CO_2 by the citric acid cycle, with the concomitant generation of energy, or incorporation into lipid (Figure 17.16). As expected of an enzyme at a critical branch point in metabolism, the activity of the pyruvate dehydrogenase complex is stringently controlled by several means (Figure 17.17). High concentrations of reaction products of the complex inhibit the reaction: acetyl CoA inhibits the transacetylase component (E_2), whereas NADH inhibits the dihydrolipoyl dehydrogenase (E_3). However, the key means of regulation in eukaryotes is covalent modification of the pyruvate dehydrogenase component. *Phos-phorylation of the pyruvate dehydrogenase component (E_1) by a specific kinase switches off the activity of the complex. Deactivation is reversed by the action of a specific phosphatase.* The site of phosphorylation is the transacetylase component (E_2), again highlighting the structural and mechanistic importance of this core. Increasing the NADH/NAD^+, acetyl CoA/CoA, or ATP/ADP ratio promotes phosphorylation and, hence, deactivation of the complex. In other words, high concentrations of immediate (acetyl CoA and NADH) and ultimate (ATP) products inhibit the activity. Thus, *pyruvate dehydrogenase is switched off when the energy charge is high and biosynthetic intermediates are abundant.* On the other hand, pyruvate as well as ADP (a signal of low energy charge) activate the dehydrogenase by inhibiting the kinase.

In contrast, α_1-adrenergic agonists and hormones such as vasopressin stimulate pyruvate dehydrogenase by triggering a rise in the cytosolic Ca^{2+} level (Section 15.3.2), which in turn elevates the mitochondrial Ca^{2+} level. The rise in mitochondrial Ca^{2+} activates the pyruvate dehydrogenase complex by stimulating the phosphatase. Insulin also accelerates the conversion of pyruvate into acetyl CoA by stimulating the dephosphorylation of the complex. In turn, glucose is funneled into pyruvate.

The importance of this covalent control is illustrated in people with a phosphatase deficiency. Because pyruvate dehydrogenase is always phosphorylated and thus inactive, glucose is processed to lactic acid. This condition results in unremitting lactic acidosis (high blood levels of lactic acid), which leads to the malfunctioning of many tissues, most notably the central nervous system (Section 17.3.2).

17.2.2. The Citric Acid Cycle Is Controlled at Several Points

The rate of the citric acid cycle is precisely adjusted to meet an animal cell's needs for ATP (Figure 17.18). The primary control points are the allosteric enzymes isocitrate dehydrogenase and α-ketoglutarate dehydrogenase.

Isocitrate dehydrogenase is allosterically stimulated by ADP, which enhances the enzyme's affinity for substrates. The binding of isocitrate, NAD^+, Mg^{2+}, and ADP is mutually cooperative. In contrast, NADH inhibits iso-citrate dehydrogenase by directly displacing NAD^+. ATP, too, is inhibitory. It is important to note that several steps in the cycle require NAD^+ or FAD, which are abundant only when the energy charge is low.

A second control site in the citric acid cycle is α-ketoglutarate dehydrogenase. Some aspects of this enzyme's control are like those of the pyruvate dehydrogenase complex, as might be expected from the homology of the two enzymes. α-Ketoglutarate dehydrogenase is inhibited by succinyl CoA and NADH, the products of the reaction that it catalyzes. In addition, α-ketoglutarate dehydrogenase is inhibited by a high energy charge. Thus, *the rate of the cycle is reduced when the cell has a high level of ATP.*

In many bacteria, the funneling of two-carbon fragments into the cycle also is controlled. *The synthesis of citrate from oxaloacetate and acetyl CoA carbon units is an important control point in these organisms.* ATP is an allosteric inhibitor of citrate synthase. The effect of ATP is to increase the value of K_M for acetyl CoA. Thus, as the level of ATP increases, less of this enzyme is saturated with acetyl CoA and so less citrate is formed.

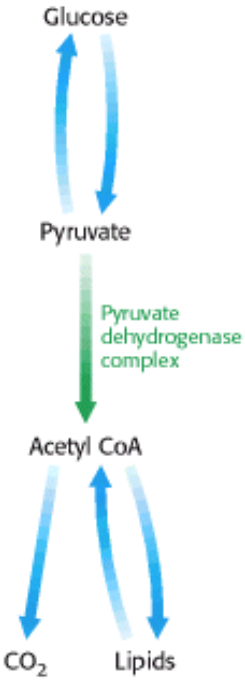


Figure 17.16. From Glucose to Acetyl CoA. The synthesis of acetyl CoA by the pyruvate dehydrogenase complex is a key irreversible step in the metabolism of glucose.

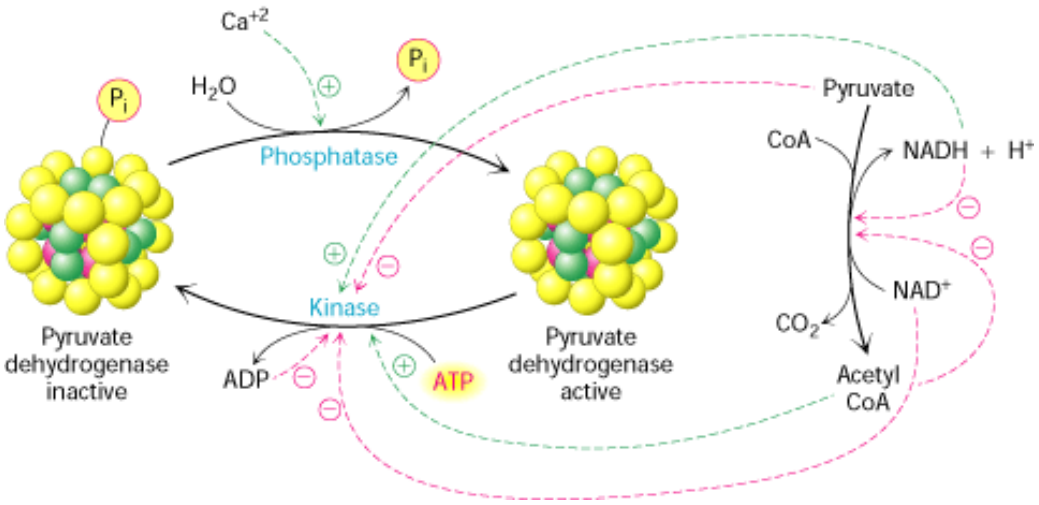


Figure 17.17. Regulation of the Pyruvate Dehydrogenase Complex. The complex is inhibited by its immediate products, NADH and acetyl CoA. The pyruvate dehydrogenase component is also regulated by covalent modification. A specific kinase phosphorylates and inactivates pyruvate dehydrogenase, and a phosphatase actives the dehydrogenase by removing the phosphoryl. The kinase and the phosphatase also are highly regulated enzymes.

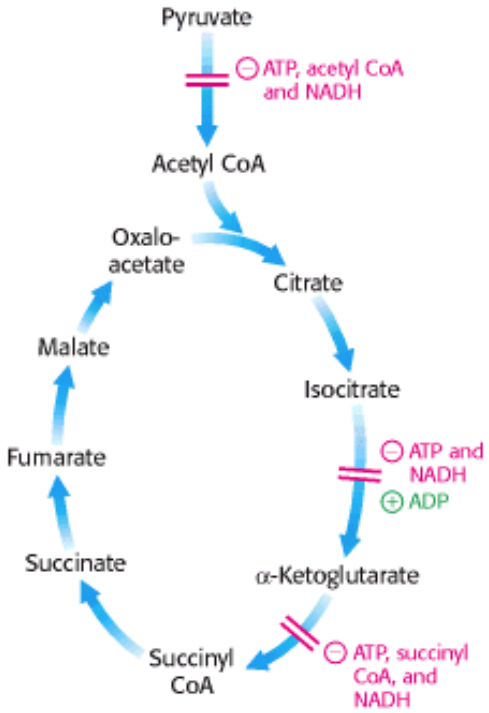


Figure 17.18. Control of the Citric Acid Cycle. The citric acid cycle is regulated primarily by the concentration of ATP and NADH. The key control points are the enzymes isocitrate dehydrogenase and α-ketoglutarate dehydrogenase.

17.3. The Citric Acid Cycle Is a Source of Biosynthetic Precursors

Thus far, discussion has focused on the citric acid cycle as the *major degradative pathway for the generation of ATP*. As a major metabolic hub of the cell, the citric acid cycle also *provides intermediates for biosyntheses* (Figure 17.19). For example, most of the carbon atoms in porphyrins come from *succinyl CoA*. Many of the amino acids are derived from α-*ketoglutarate* and *oxaloacetate*. These biosynthetic processes will be discussed in subsequent chapters.

17.3.1. The Citric Acid Cycle Must Be Capable of Being Rapidly Replenished

The important point now is that *citric acid cycle intermediates must be replenished if any are drawn off for biosyntheses*. Suppose that much oxaloacetate is converted into amino acids for protein synthesis and, subsequently, the energy needs of the cell rise. The citric acid cycle will operate to a reduced extent unless new oxaloacetate is formed, because acetyl CoA cannot enter the cycle unless it condenses with oxaloacetate. Even though oxaloacetate is recycled, a minimal level must be maintained to allow the cycle to function.

How is oxaloacetate replenished? Mammals lack the enzymes for the net conversion of acetyl CoA into oxaloacetate or any other citric acid cycle intermediate. Rather, oxaloacetate is formed by the carboxylation of pyruvate, in a reaction catalyzed by the biotin-dependent enzyme *pyruvate carboxylase*.

$$\text{Pyruvate} + CO_2 + \text{ATP} + H_2O \longrightarrow \text{oxaloacetate} + \text{ADP} + P_i + 2\,H^+$$

Recall that this enzyme plays a crucial role in gluconeogenesis (Section 16.3.2). It is active only in the presence of acetyl CoA, which signifies the need for more oxaloacetate. If the energy charge is high, oxaloacetate is converted into glucose. If the energy charge is low, oxaloacetate replenishes the citric acid cycle. The synthesis of oxaloacetate by the carboxylation of pyruvate is an example of an *anaplerotic reaction* (of Greek origin, meaning to "fill up"), a reaction that leads to the net synthesis, or replenishment, of pathway components. Note that, because the citric acid cycle is a cycle, it can be replenished by the generation of any of the intermediates.

17.3.2. The Disruption of Pyruvate Metabolism Is the Cause of Beriberi and Poisoning by Mercury and Arsenic

Beriberi, a neurologic and cardiovascular disorder, is caused by a dietary deficiency of thiamine (also called *vitamin* B_1). The disease has been and continues to be a serious health problem in the Far East because rice, the major food, has a rather low content of thiamine. This deficiency is partly ameliorated if the whole rice grain is soaked in water before milling—some of the thiamine in the husk then leaches into the rice kernel. The problem is exacerbated if the rice is polished, because only the outer layer contains significant amounts of thiamine. Beriberi is also occasionally seen in alcoholics who are severely malnourished and thus thiamine deficient. The disease is characterized by neurologic and cardiac symptoms. Damage to the peripheral nervous system is expressed as pain in the limbs, weakness of the musculature, and distorted skin sensation. The heart may be enlarged and the cardiac output inadequate.

Beriberi-

A vitamin-deficiency disease first described in 1630 by Jacob Bonitus, a Dutch physician working in Java:

"A certain very troublesome affliction, which attacks men, is called by the inhabitants Beriberi (which means sheep). I believe those, whom this same disease attacks, with their knees shaking and the legs raised up, walk like sheep. It is a kind of paralysis, or rather Tremor: for it penetrates the motion and sensation of the hands and feet indeed sometimes of the whole body."

Which biochemical processes might be affected by a deficiency of thiamine? *Thiamine pyrophosphate is the prosthetic group of three important enzymes: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and transketolase.* Transketolase functions in the pentose phosphate pathway, which will be discussed in Chapter 20. *The common feature of enzymatic reactions utilizing TPP is the transfer of an activated aldehyde unit.* In beriberi, the levels of pyruvate and α-ketoglutarate in the blood are higher than normal. The increase in the level of pyruvate in the blood is especially pronounced after the ingestion of glucose. A related finding is that the activities of the pyruvate and α-ketoglutarate dehydrogenase complexes in vivo are abnormally low. The low transketolase activity of red cells in beriberi is an easily measured and reliable diagnostic indicator of the disease.

Why does TPP deficiency lead primarily to neurological disorders? The nervous system relies essentially on glucose as its only fuel. In contrast, most other tissues can use fats as a source of fuel for the citric acid cycle. The product of aerobic glycolysis, pyruvate, can enter the citric acid cycle only through the pyruvate dehydrogenase complex.

Symptoms similar to those of beriberi arise if an organism is exposed to mercury or arsenite (AsO_3^{3-}). Both elements have a high affinity for neighboring sulfhydryls, such as those in the reduced dihydrolipoyl groups of the dihydrolipoyl dehydrogenase component of the pyruvate dehydrogenase complex (Figure 17.20). The binding of mercury or arsenite to the dihydrolipoyl groups inhibits the complex and leads to central nervous system pathologies. The proverbial phrase "mad as a hatter" refers to the strange behavior of poisoned hat makers who used mercury nitrate to soften and shape animal furs. This form of mercury is absorbed through the skin. Similar problems afflicted the early photographers, who used vaporized mercury to create daguerreotypes.

[The Granger Collection.]

Treatment for these poisons is the administration of sulfhydryl reagents with adjacent sulfhydryl groups to compete with the dihydrolipoyl residues for binding with the metal ion, which is then excreted in the urine. Indeed, 2,3-dimercaptopropanol (see Figure 17.20) was developed after World War I as an antidote to lewisite, an arsenic-based chemical weapon. This compound was initially called BAL, for British anti-lewisite.

17.3.3. Speculations on the Evolutionary History of the Citric Acid Cycle

How did the citric acid cycle come into being? Although definitive answers are elusive, it is nevertheless instructive to speculate how this complicated central hub of metabolism developed. We can perhaps begin to comprehend how evolution might work at the level of biochemical pathways.

The manuscript proposing the citric acid cycle was submitted for publication to *Nature* but was rejected. It was subsequently published in *Enzymologia*. Dr. Krebs proudly displayed the rejection letter throughout his career as encouragement for young scientists.

"June 1937

The editor of NATURE presents his compliments to Dr. H. A. Krebs and regrets that as he has already sufficient letters to fill the correspondence columns of NATURE for seven or eight weeks, it is undesirable to accept further letters at the present time on account of the time delay which must occur in their publication.

If Dr. Krebs does not mind much delay the editor is prepared to keep the letter until the congestion is relieved in the hope of making use of it.

He returns it now, in case Dr. Krebs prefers to submit it for early publication to another periodical."

It is most likely that the citric acid cycle was assembled from preexisting reaction pathways. As noted earlier, many of the intermediates formed in the citric acid cycle are used in biosynthetic pathways to generate amino acids and porphyrins. Thus, compounds such as pyruvate, α-ketoglutarate, and oxaloacetate were likely present early in evolution for biosynthetic purposes. The oxidative decarboxylation of these α-ketoacids is quite favorable thermodynamically. The elegant modular structures of the pyruvate and α-ketoglutarate dehydrogenase complexes reveal how three reactions (decarboxylation, oxidation, and thioester formation) can be linked to harness the free energy associated with decarboxylation to drive the synthesis of both acyl CoA derivatives and NADH. These reactions almost certainly formed the core of processes that preceded the citric acid cycle evolutionarily. Interestingly, α-ketoglutarate can be directly converted into oxaloacetate by transamination of the respective amino acids by aspartate aminotransferase, another key biosynthetic enzyme. Thus, cycles comprising smaller numbers of intermediates could have existed before the present form evolved to harvest the electrons from pyruvate or other compounds more efficiently.

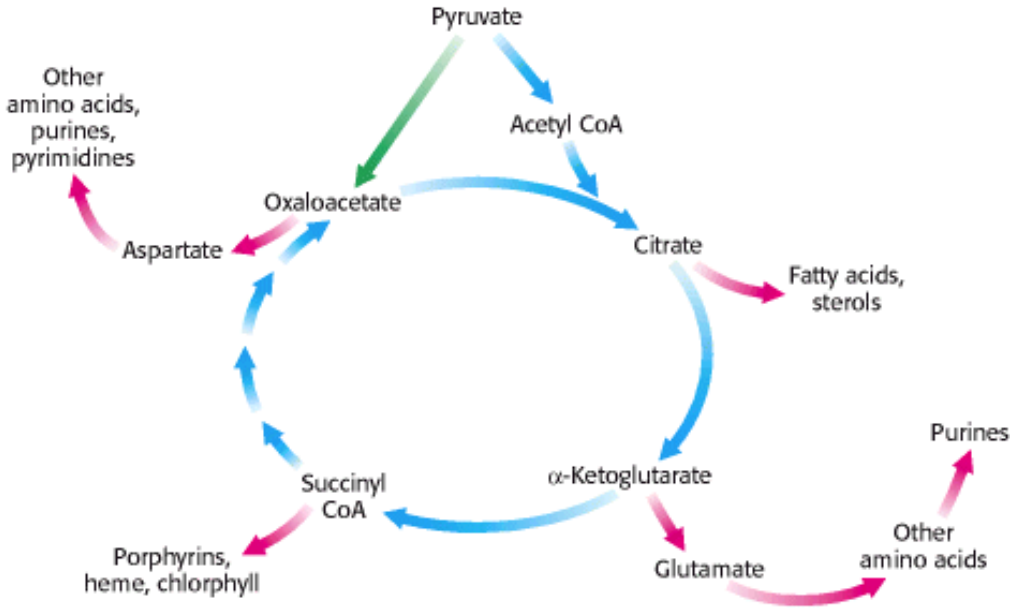


Figure 17.19. Biosynthetic Roles of the Citric Acid Cycle. Intermediates drawn off for biosyntheses (shown by red arrows) are replenished by the formation of oxaloacetate from pyruvate.

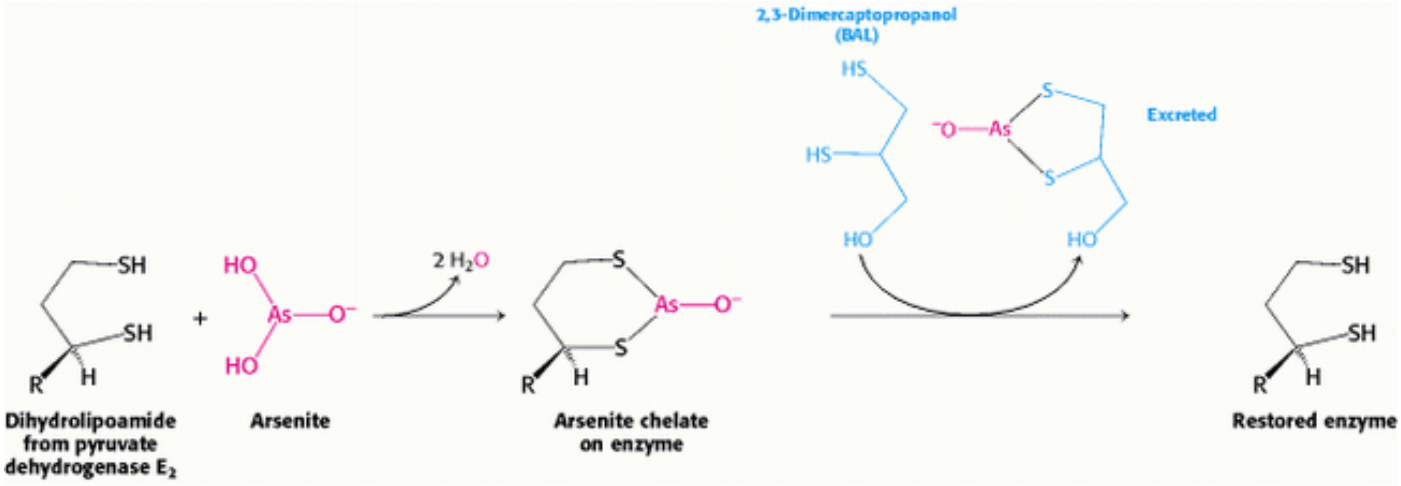


Figure 17.20. Arsenite Poisoning. Arsenite inhibits the pyruvate dehydrogenase complex by inactivating the dihydrolipoamide component of the transacetylase. Some sulfhydryl reagents, such as 2,3-dimercaptoethanol, relieve the inhibition by forming a complex with the arsenite that can be excreted.

17.4. The Glyoxylate Cycle Enables Plants and Bacteria to Grow on Acetate

Many bacteria and plants are able to subsist on acetate or other compounds that yield acetyl CoA. They make use of a metabolic pathway absent in most other organisms that converts two-carbon acetyl units into four-carbon units (succinate) for energy production and biosyntheses. This reaction sequence, called the *glyoxylate cycle*, bypasses the two decarboxylation steps of the citric acid cycle. Another key difference is that two molecules of acetyl CoA enter per turn of the glyoxylate cycle, compared with one in the citric acid cycle.

The glyoxylate cycle (Figure 17.21), like the citric acid cycle, begins with the condensation of acetyl CoA and oxaloacetate to form citrate, which is then isomerized to isocitrate. Instead of being decarboxylated, isocitrate is cleaved by *isocitrate lyase* into succinate and glyoxylate. The subsequent steps regenerate oxaloacetate from glyoxylate. Acetyl CoA condenses with glyoxylate to form malate in a reaction catalyzed by *malate synthase*, which resembles citrate synthase. Finally, malate is oxidized to oxaloacetate, as in the citric acid cycle. The sum of these reactions is:

$$2\,\text{Acetyl CoA} + \text{NAD}^+ + 2\,\text{H}_2\text{O} \longrightarrow \text{succinate} + 2\,\text{CoA} + \text{NADH} + 2\,\text{H}^+$$

In plants, these reactions take place in organelles called *glyoxysomes.* Succinate, released midcycle, can be converted into carbohydrates by a combination of the citric acid cycle and gluconeogenesis. Thus, organisms with the glyoxylate cycle gain a metabolic versatility.

Bacteria and plants can synthesize acetyl CoA from acetate and CoA by an ATP-driven reaction that is catalyzed by *acetyl CoA synthetase.*

$$\text{Acetate} + \text{CoA} + \text{ATP} \longrightarrow \text{acetyl CoA} + \text{AMP} + \text{PP}_i$$

Pyrophosphate is then hydrolyzed to orthophosphate, and so the equivalents of two compounds having high phosphoryl transfer potential are consumed in the activation of acetate. We will return to this type of activation reaction in fatty acid degradation (Section 22.2.2), where it is used to form fatty acyl CoA, and in protein synthesis, where it is used to link amino acids to transfer RNAs (Section 29.2.1).

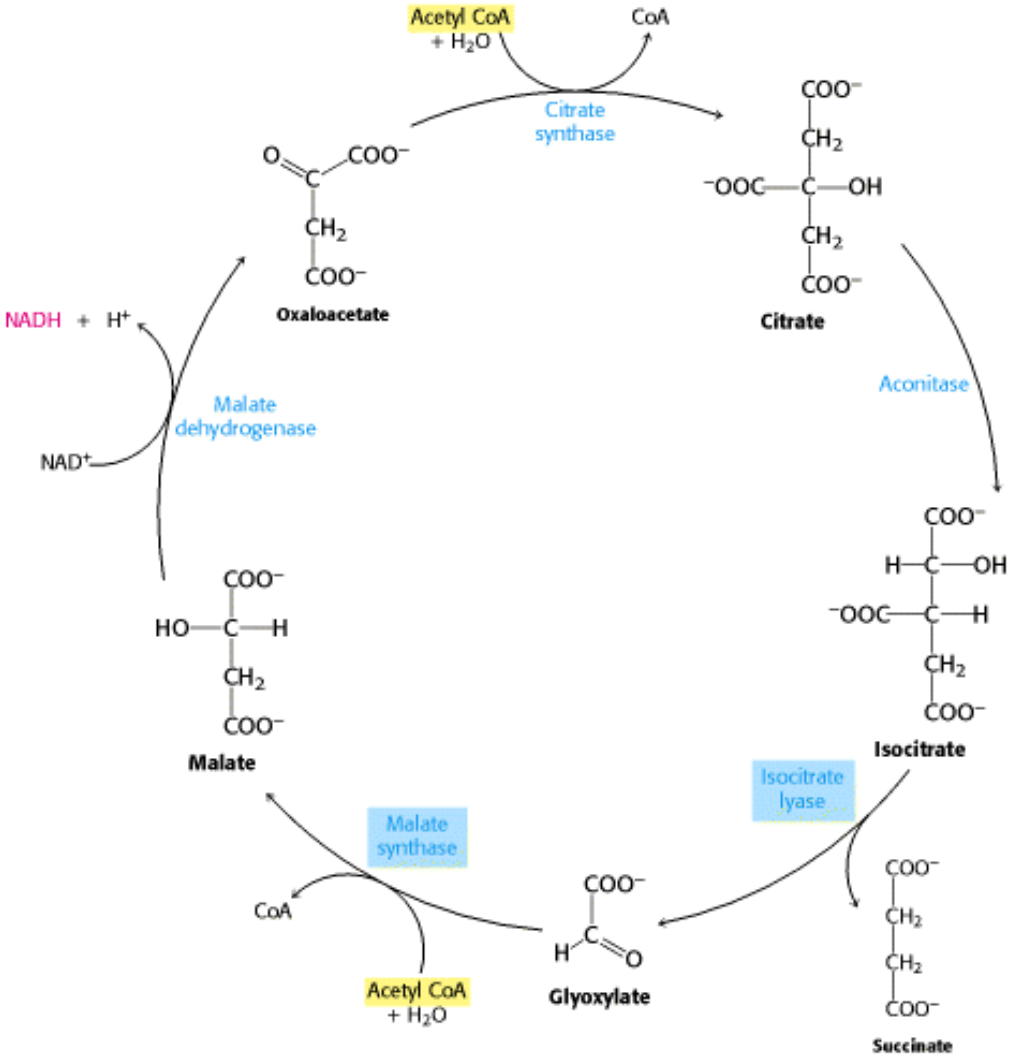


Figure 17.21. The Glyoxylate Pathway. The glyoxylate cycle allows plants and some microorganisms to grow on acetate because the cycle bypasses the decarboxylation steps of the citric acid cycle. The enzymes that permit the

conversion of acetate into succinate-isocitrate lyase and malate synthase-are boxed in blue.

Summary

The citric acid cycle is the final common pathway for the oxidation of fuel molecules. It also serves as a source of building blocks for biosyntheses. Most fuel molecules enter the cycle as acetyl CoA. The link between glycolysis and the citric acid cycle is the oxidative decarboxylation of pyruvate to form acetyl CoA. In eukaryotes, this reaction and those of the cycle take place inside mitochondria, in contrast with glycolysis, which takes place in the cytosol.

The Citric Acid Cycle Oxidizes Two-Carbon Units

The cycle starts with the condensation of oxaloacetate (C_4) and acetyl CoA (C_2) to give citrate (C_6), which is isomerized to isocitrate (C_6). Oxidative decarboxylation of this intermediate gives α-ketoglutarate (C_5). The second molecule of carbon dioxide comes off in the next reaction, in which α-ketoglutarate is oxidatively decarboxylated to succinyl CoA (C_4). The thioester bond of succinyl CoA is cleaved by inorthophosphate to yield succinate, and a high phosphoryl transfer potential compound in the form of GTP is concomitantly generated. Succinate is oxidized to fumarate (C_4), which is then hydrated to form malate (C_4). Finally, malate is oxidized to regenerate oxaloacetate (C_4). Thus, two carbon atoms from acetyl CoA enter the cycle, and two carbon atoms leave the cycle as CO_2 in the successive decarboxylations catalyzed by isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. In the four oxidation-reduction reactions in the cycle, three pairs of electrons are transferred to NAD^+ and one pair to FAD. These reduced electron carriers are subsequently oxidized by the electron-transport chain to generate approximately 9 molecules of ATP. In addition, 1 molecule of a compound having a high phosphoryl transfer potential is directly formed in the citric acid cycle. Hence, a total of 10 molecules of compounds having high phosphoryl transfer potential are generated for each two-carbon fragment that is completely oxidized to H_2O and CO_2.

Entry to the Citric Acid Cycle and Metabolism Through It Are Controlled

The citric acid cycle operates only under aerobic conditions because it requires a supply of NAD^+ and FAD. The irreversible formation of acetyl CoA from pyruvate is an important regulatory point for the entry of glucose-derived pyruvate into the citric acid cycle. The activity of the pyruvate dehydrogenase complex is stringently controlled by reversible phosphorylation. The electron acceptors are regenerated when NADH and $FADH_2$ transfer their electrons to O_2 through the electron-transport chain, with the concomitant production of ATP. Consequently, the rate of the citric acid cycle depends on the need for ATP. In eukaryotes, the regulation of two enzymes in the cycle also is important for control. A high energy charge diminishes the activities of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. These mechanisms complement each other in reducing the rate of formation of acetyl CoA when the energy charge of the cell is high and when biosynthetic intermediates are abundant.

The Citric Acid Cycle Is a Source of Biosynthetic Precursors

When the cell has adequate energy available, the citric acid cycle can also provide a source of building blocks for a host of important biomolecules, such as nucleotide bases, proteins, and heme groups. This use depletes the cycle of intermediates. When the cycle again needs to metabolize fuel, anaplerotic reactions replenish the cycle intermediates.

The Glyoxylate Cycle Enables Plants and Bacteria to Grow on Acetate

The glyoxylate cycle enhances the metabolic versatility of many plants and bacteria. This cycle, which uses some of the reactions of the citric acid cycle, enables these organisms to subsist on acetate because it bypasses the two decarboxylation steps of the citric acid cycle.

Key Terms

citric acid (tricarboxylic acid, TCA; Krebs) cycle

oxidative phosphorylation

acetyl CoA

pyruvate dehydrogenase complex

flavoprotein

citrate synthase

iron-sulfur (nonheme iron) protein

isocitrate dehydrogenase

α-ketoglutarate dehydrogenase

metabolon

anaplerotic reaction

beriberi

glyoxylate cycle

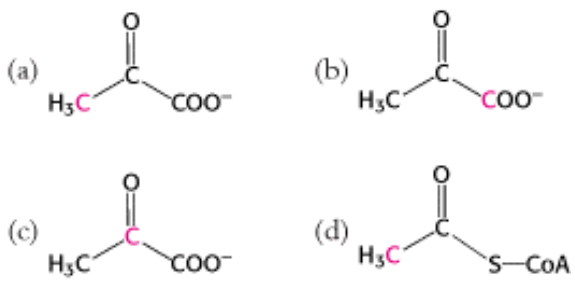
isocitrate lyase

malate synthase

glyoxysome

Problems

1. *Flow of carbon atoms.* What is the fate of the radioactive label when each of the following compounds is added to a cell extract containing the enzymes and cofactors of the glycolytic pathway, the citric acid cycle, and the pyruvate dehydrogenase complex? (The ^{14}C label is printed in red.)



(e) Glucose 6-phosphate labeled at C-1.

See answer

2.

$$C_2 + C_2 \longrightarrow C_4$$

(a) Which enzymes are required to get *net synthesis* of oxaloacetate from acetyl CoA?

(b) Write a balanced equation for the net synthesis.

(c) Do mammalian cells contain the requisite enzymes?

See answer

3. *Driving force.* What is the $\Delta G^{\circ\prime}$ for the complete oxidation of the acetyl unit of acetyl CoA by the citric acid cycle?

See answer

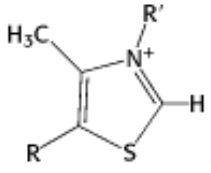
4. *Acting catalytically.* The citric acid cycle itself, which is composed of enzymatically catalyzed steps, can be thought of essentially as the product of a supramolecular enzyme. Explain.

See answer

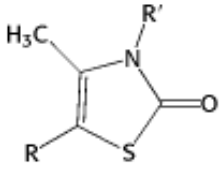
5. *Probing stereospecificity.* A sample of deuterated reduced NAD was prepared by incubating $H_3C\text{-}CD_2\text{-}OH$ and NAD^+ with alcohol dehydrogenase. This reduced coenzyme was added to a solution of 1,3-BPG and glyceraldehyde 3-phosphate dehydrogenase. The NAD^+ formed by this second reaction contained one atom of deuterium, whereas glyceraldehyde 3-phosphate, the other product, contained none. What does this experiment reveal about the stereospecificity of glyceraldehyde 3-phosphate dehydrogenase?

See answer

6. *A potent inhibitor.* Thiamine thiazolone pyrophosphate binds to pyruvate dehydrogenase about 20,000 times as strongly as does thiamine pyrophosphate, and it competitively inhibits the enzyme. Why?



TPP



Thiazolone analog of TPP

See answer

7. *Lactic acidosis.* Patients in shock will often suffer from lactic acidosis due to a deficiency of O_2. Why does a lack of O_2 lead to lactic acid accumulation? One treatment for shock is to administer dichloroacetate, which inhibits the kinase associated with the pyruvate dehydrogenase complex. What is the biochemical rationale for this treatment?

See answer

8. *Coupling reactions.* The oxidation of malate by NAD^+ to form oxaloacetate is a highly endergonic reaction under standard conditions [$\Delta G^{\circ\prime}$ + 7 kcal mol^{-1} (+ 29 kJ mol^{-1})]. The reaction proceeds readily under physiological conditions.

(a) Why?

(b) Assuming an $[NAD^+]/[NADH]$ ratio of 8 and a pH of 7, what is the lowest [malate]/[oxaloacetate] ratio at which oxaloacetate can be formed from malate?

See answer

9. *Synthesizing α-ketoglutarate.* It is possible, with the use of the reactions and enzymes discussed in this chapter, to convert pyruvate into α-ketoglutarate without depleting any of the citric acid cycle components. Write a balanced reaction scheme for this conversion, showing cofactors and identifying the required enzymes.

See answer

Chapter Integration Problem

10. *Fats into glucose?* Fats are usually metabolized into acetyl CoA and then further processed through the citric acid cycle. In Chapter 16, we learned that glucose could be synthesized from oxaloacetate, a citric acid cycle intermediate. Why, then, after a long bout of exercise depletes our carbohydrate stores, do we need to replenish those stores by eating carbohydrates? Why do we not simply replace them by converting fats into carbohydrates?

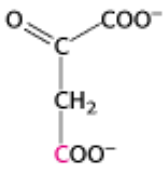
See answer

Mechanism Problems

11. *Theme and variation.* Propose a reaction mechanism for the condensation of acetyl CoA and glyoxylate in the glyoxylate cycle of plants and bacteria.

See answer

12. *Symmetry problems.* In experiments carried out in 1941 to investigate the citric acid cycle, oxaloacetate labeled with ^{14}C in the carboxyl carbon atom farthest from the keto group was introduced to an active preparation of mitochondria.



Oxaloacetate

Analysis of the α-ketoglutarate formed showed that none of the radioactive label had been lost. Decarboxylation of α-ketoglutarate then yielded succinate devoid of radioactivity. All the label was in the released CO_2. Why were the early investigators of the citric acid cycle surprised that *all* the label emerged in the CO_2?

See answer

13. *Symmetric molecules reacting asymmetrically.* The interpretation of the experiments described in problem 12 was that citrate (or any other symmetric compound) cannot be an intermediate in the formation of α-ketoglutarate, because of the asymmetric fate of the label. This view seemed compelling until Alexander Ogston incisively pointed out in 1948 that "it is possible that *an asymmetric enzyme which attacks a symmetrical compound can distinguish between its identical groups.*" For simplicity, consider a molecule in which two hydrogen atoms, a group X, and a different group Y are bonded to a tetrahedral carbon atom as a model for citrate. Explain how a symmetric molecule can react with an enzyme in an asymmetric way.

See answer

Data Interpretation

14. *A little goes a long way.* As will become clearer in Chapter 18, the activity of the citric acid cycle can be monitored by monitoring the amount of O_2 consumed. The greater the rate of O_2 consumption, the faster the rate of the cycle. Hans Krebs used this assay to investigate the cycle in 1937. He used as his experimental system minced pigeon breast muscle, which is rich in mitochondria. In one set of experiments, Krebs measured the O_2 consumption in the presence of carbohydrate only and in the presence of carbohydrate and citrate. The results are shown in the following table.

(a) How much O_2 would be absorbed if the added citrate were completely oxidized to H_2O and CO_2?

(b) Based on your answer to part *a*, what do the results given in the table suggest?

See answer

15. *Arsenite poisoning*. The effect of arsenite on the experimental system of problem 14 was then examined. Experimental data (not presented here) showed that the amount of citrate present did not change in the course of the experiment in the absence of arsenite. However, if arsenite was added to the system, different results were obtained, as shown in the following table.

(a) What is the effect of arsenite on the disappearance of citrate?

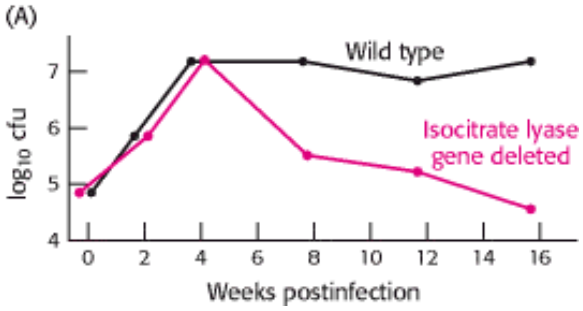
(b) How is the arsenite's action altered by the addition of more citrate?

(c) What do these data suggest about the site of action of arsenite?

See answer

16. *Isocitrate lyase and tuberculosis.* The bacterium *Mycobacterium tuberculosis,* the cause of tuberculosis, can invade the lungs and persist in a latent state for years. During this time, the bacteria reside in granulomas—nodular scars containing bacteria and host-cell debris in the center and surrounded by immune cells. The granulomas are lipid-rich, oxygen-poor environments. How these bacteria manage to persist is something of a mystery. The results of recent research suggest that the glyoxylate cycle is required for the persistence. The following data show the amount of bacteria [presented as colony-forming units (cfu)] in mice lungs in the weeks after an infection.

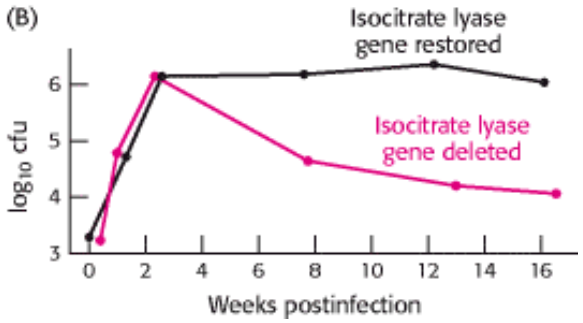
In graph A, the black circles represent the results for wild-type bacteria and the red circles represent the results for bacteria from which the gene for isocitrate lyase was deleted.



(a) What is the effect of the absence of isocitrate lyase?

The techniques discussed in Chapter 6 were used to reinsert the gene encoding isocitrate lyase into bacteria from which it had previously been deleted.

In graph B, black circles represent bacteria into which the gene was reinserted and red circles bacteria from which the gene was still missing.



[Data after McKinney et al., 2000. *Nature* 406:735–738.]

(b) Do these results support those obtained in part *a*?

(c) What is the purpose of the experiment in part *b*?

(d) Why do these bacteria perish in the absence of the glyoxylate cycle?

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See answer

Effect of citrate on oxygen consumption by minced pigeon breast muscle

Time (min)	Micromoles of oxygen consumed	
	Carbohydrate only	Carbohydrate plus 3 μmol of citrate
10	26	28
60	43	62
90	46	77
150	49	85

Disappearance of citric acid in pigeon breast muscle in the presence of arsenite

Micromoles of citrate added	Micromoles of citrate found after 40 minutes	Micromoles of citrate used
22	0.6	21
44	20	24
90	56	34

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Where to start

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18. Oxidative Phosphorylation

The NADH and $FADH_2$ formed in glycolysis, fatty acid oxidation, and the citric acid cycle are energy-rich molecules because each contains a pair of electrons having a high transfer potential. When these electrons are used to reduce molecular oxygen to water, a large amount of free energy is liberated, which can be used to generate ATP. *Oxidative phosphorylation is the process in which ATP is formed as a result of the transfer of electrons from NADH or* $FADH_2$ *to* O_2 *by a series of electron carriers.* This process, which takes place in mitochondria, is the major source of ATP in aerobic organisms (Figure 18.1). For example, oxidative phosphorylation generates 26 of the 30 molecules of ATP that are formed when glucose is completely oxidized to CO_2 and H_2O.

Oxidative phosphorylation is conceptually simple and mechanistically complex. Indeed, the unraveling of the mechanism of oxidative phosphorylation has been one of the most challenging problems of biochemistry. The flow of electrons from NADH or $FADH_2$ to O_2 through protein complexes located in the mitochondrial inner membrane leads to the pumping of protons out of the mitochondrial matrix. The resulting uneven distribution of protons generates a pH gradient and a transmembrane electrical potential that creates a *proton-motive force*. ATP is synthesized when protons flow back to the mitochondrial matrix through an enzyme complex. Thus, *the oxidation of fuels and the phosphorylation of ADP are coupled by a proton gradient across the inner mitochondrial membrane* (Figure 18.2).

Oxidative phosphorylation is the culmination of a series of energy transformations that are called *cellular respiration* or simply *respiration* in their entirety. First, carbon fuels are oxidized in the citric acid cycle to yield electrons with high transfer potential. Then, this electron-motive force is converted into a proton-motive force and, finally, the proton-motive force is converted into phosphoryl transfer potential. The conversion of electron-motive force into proton-motive force is carried out by three electron-driven proton pumps—NADH-Q oxidoreductase, Q-cytochrome *c* oxidoreductase, and

cytochrome *c* oxidase. These large transmembrane complexes contain multiple oxidation-reduction centers, including quinones, flavins, iron-sulfur clusters, hemes, and copper ions. The final phase of oxidative phosphorylation is carried out by *ATP synthase,* an ATP-synthesizing assembly that is driven by the flow of protons back into the mitochondrial matrix. Components of this remarkable enzyme rotate as part of its catalytic mechanism. Oxidative phosphorylation vividly shows that *proton gradients are an interconvertible currency of free energy in biological systems.*

Respiration

An ATP-generating process in which an inorganic compound (such as molecular oxygen) serves as the ultimate electron acceptor. The electron donor can be either an organic compound or an inorganic one.

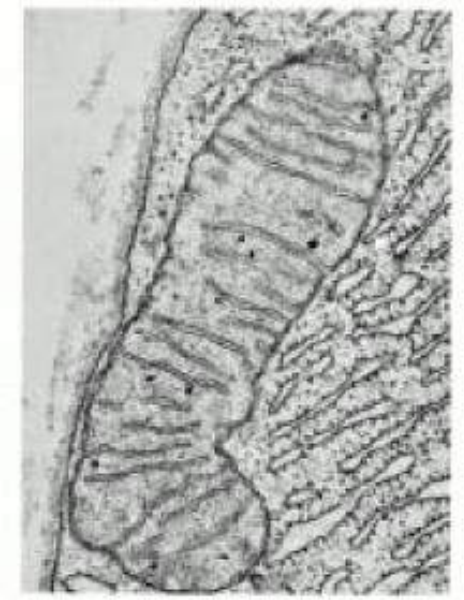


Figure 18.1. Electron Micrograph of a Mitochondrion. [Courtesy of Dr. George Palade.]

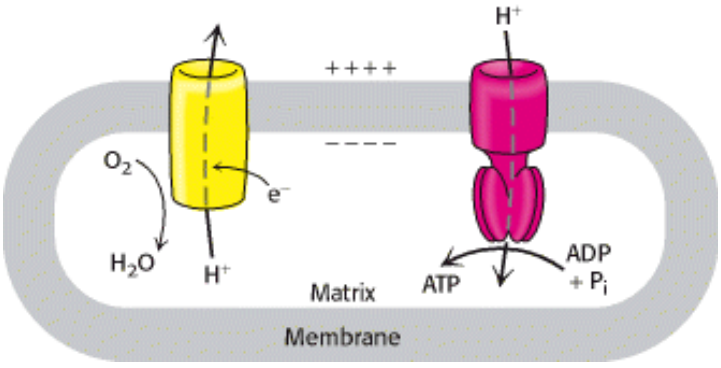
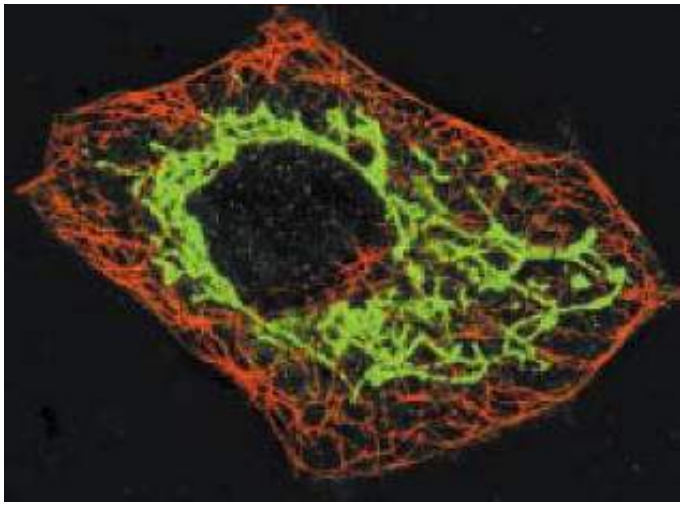
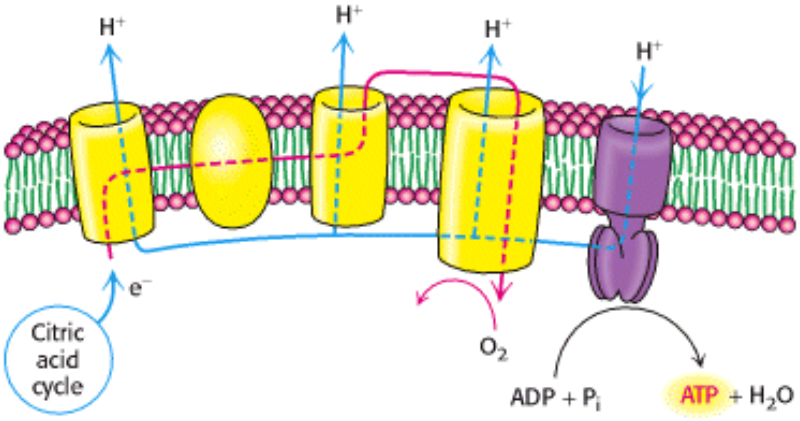


Figure 18.2. Essence of Oxidative Phosphorylation. Oxidation and ATP synthesis are coupled by transmembrane proton fluxes.





Mitochondria, Stained Green, Form a Network Inside a Fibroblast Cell (Left). Mitochondria oxidize carbon fuels to form cellular energy. This transformation requires electron transfer through several large protein complexes (above), some of which pump protons, forming a proton gradient that powers the synthesis of ATP. [(Left) Courtesy of Michael P. Yaffee, Dept. of Biology, University of California at San Diego.]

18.1. Oxidative Phosphorylation in Eukaryotes Takes Place in Mitochondria

Mitochondria are oval-shaped organelles, typically about 2 μm in length and 0.5 μm in diameter, about the size of a bacterium. Eugene Kennedy and Albert Lehninger discovered a half-century ago that *mitochondria contain the respiratory assembly, the enzymes of the citric acid cycle, and the enzymes of fatty acid oxidation.*

18.1.1. Mitochondria Are Bounded by a Double Membrane

Electron microscopic studies by George Palade and Fritjof Sjöstrand revealed that mitochondria have two membrane systems: an *outer membrane* and an extensive, highly folded *inner membrane.* The inner membrane is folded into a series of internal ridges called *cristae.* Hence, there are two compartments in mitochondria: (1) the *intermembrane space* between the outer and the inner membranes and (2) the *matrix,* which is bounded by the inner membrane (Figure 18.3). Oxidative phosphorylation takes place in the inner mitochondrial membrane, in contrast with most of the reactions of the citric acid cycle and fatty acid oxidation, which take place in the matrix.

The outer membrane is quite permeable to most small molecules and ions because it contains many copies of *mitochondrial porin,* a 30–35 kd poreforming protein also known as VDAC, for voltage-dependent anion channel.

VDAC plays a role in the regulated flux of metabolites—usually anionic species such as phosphate, chloride, organic anions, and the adenine nucleotides—across the outer membrane. VDAC appears to form an open β -barrel structure similar to that of the bacterial porins (Section 12.5.2), although mitochondrial porins and bacterial porins may have evolved independently. Some cytoplasmic kinases bind to VDAC, thereby obtaining preferential access to the exported ATP. In contrast, the inner membrane is intrinsically impermeable to nearly all ions and polar molecules. A large family of transporters shuttles metabolites such as ATP, pyruvate, and citrate across the inner mitochondrial membrane. The two faces of this membrane will be referred to as the *matrix side* and the *cytosolic side* (the latter because it is freely accessible to most small molecules in the cytosol). They are also called the *N* and *P* sides, respectively, because the membrane potential is negative on the matrix side and positive on the cytosolic side.

In prokaryotes, the electron-driven proton pumps and ATP-synthesizing complex are located in the cytoplasmic membrane, the inner of two membranes. The outer membrane of bacteria, like that of mitochondria, is permeable to most small metabolites because of the presence of porins.

18.1.2. Mitochondria Are the Result of an Endosymbiotic Event

Mitochondria are semiautonomous organelles that live in an endosymbiotic relation with the host cell. These organelles contain their own DNA, which encodes a variety of different proteins and RNAs. The genomes of mitochondrial range broadly in size across species. The mitochondrial genome of the protist *Plasmodium falciparum* consists of fewer than 6000 base pairs (6 kbp), whereas those of some land plants comprise more than 200 kbp (Figure 18.4). Human mitochondrial DNA comprises 16,569 bp and encodes 13 respiratory-chain proteins as well as the small and large ribosomal RNAs and enough tRNAs to translate all codons. However, mitochondria also contain many proteins encoded by nuclear DNA. Cells that contain mitochondria depend on these organelles for oxidative phosphorylation, and the mitochondria in turn depend on the cell for their very existence. How did this intimate symbiotic relation come to exist?

An *endosymbiotic event* is thought to have occurred whereby a freeliving organism capable of oxidative phosphorylation was engulfed by another cell. The double membrane, circular DNA (with some exceptions), and mitochondrial-specific transcription and translation machinery all point to this conclusion. Thanks to the rapid accumulation of sequence data for mitochondrial and bacterial genomes, it is now possible to speculate on the origin of the "original" mitochondrion with some authority. The most mitochondrial-like bacterial genome is that of *Rickettsia prowazekii*, the cause of louse-borne typhus. The genome for this organism is more than 1 million base pairs in size and contains 834 protein-encoding genes. Sequence data suggest that all extant mitochondria are derived from an ancestor of *R. prowazekii* as the result of a single endosymbiotic event.

The evidence that modern mitochondria result from a single event comes from examination of the most bacteria-like mitochondrial genome, that of the protozoan *Reclinomonas americana*. Its genome contains 97 genes, of which 62 specify proteins that include all of the protein-coding genes found in all of the sequenced mitochondrial genomes (Figure 18.5). Yet, this genome encodes less than 2% of the protein-coding genes in the bacterium *E. coli*. It seems unlikely that mitochondrial genomes resulting from several endosymbiotic events could have been independently reduced to the same set of genes found in *R. americana*.

Note that transient engulfment of prokaryotic cells by larger cells is not uncommon in the microbial world. In regard to mitochondria, such a transient relation became permanent as the bacterial cell lost DNA, making it incapable of independent living, and the host cell became dependent on the ATP generated by its tenant.

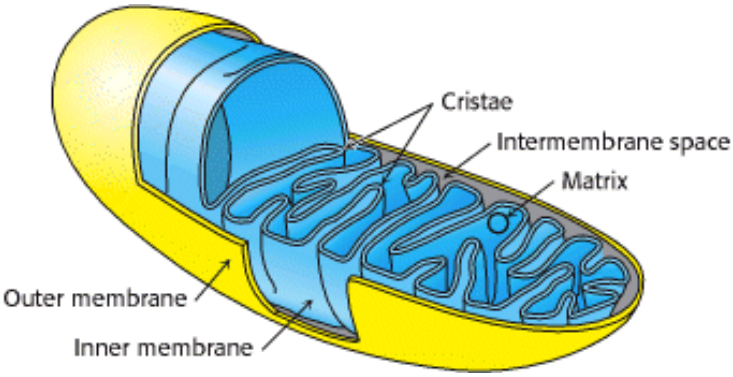


Figure 18.3. Diagram of a Mitochondrion. [After *Biology of the Cell* by Stephen L. Wolfe. © 1972 by Wadsworth Publishing Company, Inc., Belmost, California 94002. Adapted by permission of the publisher.]

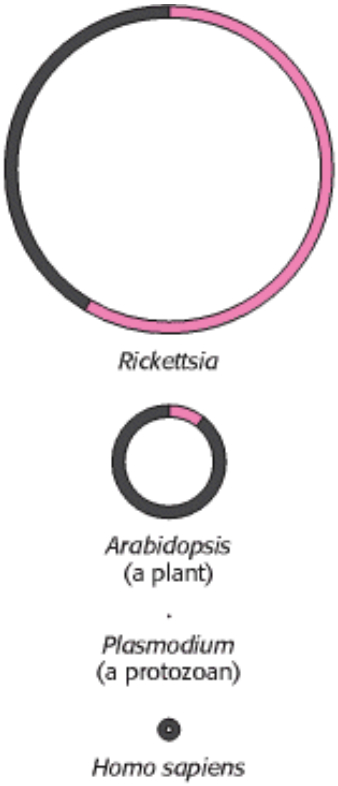


Figure 18.4. Sizes of Mitochondrial Genomes. The sizes of three mitochondrial genomes compared with the genome of *Rickettsia*, a relative of the the presumed ancestor of all mitochondria. For genomes of more than 60 kbp, the DNA coding region for genes with known function is shown in red.

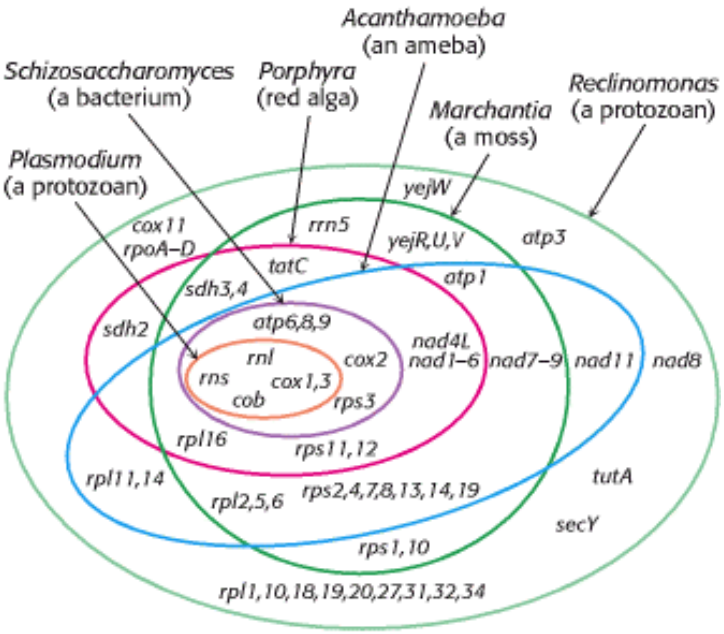


Figure 18.5. Overlapping Gene Complements of Mitochondria. The genes present within each oval are those present within the organism represented by the oval. Only rRNA- and protein-coding genes are shown. The genome of *Reclinomonas* contains all the protein-coding genes found in all the sequenced mitochondrial genomes. [After M. W. Gray, G. Burger, and B. F. Lang. *Science* 283(1999): 1476–1481.]

18.2. Oxidative Phosphorylation Depends on Electron Transfer

In Chapter 17, the primary function of the citric acid cycle was identified as the generation of NADH and $FADH_2$ by the oxidation of acetyl CoA. In oxidative phosphorylation, NADH and $FADH_2$ are used to reduce molecular oxygen to water. The highly exergonic reduction of molecular oxygen by NADH and $FADH_2$ occurs in a number of electron-transfer reactions, taking place in a set of membrane proteins known as the *electron-transport chain.*

18.2.1. High-Energy Electrons: Redox Potentials and Free-Energy Changes

High-energy electrons and redox potentials are of fundamental importance in oxidative phosphorylation. In oxidative phosphorylation, the *electron transfer potential* of NADH or $FADH_2$ is converted into the *phosphoryl transfer potential* of ATP. We need quantitative expressions for these forms of free energy. The measure of phosphoryl transfer potential is already familiar to us: it is given by $\Delta G^{\circ\prime}$ for the hydrolysis of the activated phosphate compound. The corresponding expression for the electron transfer potential is E'_0, the *reduction potential* (also called the *redox potential* or *oxidation-reduction potential*).

The reduction potential is an electrochemical concept. Consider a substance that can exist in an oxidized form X and a reduced form X^-. Such a pair is called a *redox couple*. The reduction potential of this couple can be determined by measuring the electromotive force generated by a *sample half-cell* connected to a *standard reference half-cell* (Figure 18.6). The sample half-cell consists of an electrode immersed in a solution of 1 M oxidant (X) and 1 M reductant (X^-). The standard reference half-cell consists of an electrode immersed in a 1 M H^+ solution that is in equilibrium with H_2 gas at 1 atmosphere pressure. The electrodes are connected to a voltmeter, and an agar bridge establishes electrical continuity between the half-cells. Electrons then flow from one half-cell to the other. If the reaction proceeds in the direction

$$X^- + H^+ \longrightarrow X + \tfrac{1}{2}H_2$$

the reactions in the half-cells (referred to as *half-reactions* or *couples*) must be

$$X^- \longrightarrow X + e^- \qquad H^+ + e^- \longrightarrow \tfrac{1}{2}H_2$$

Thus, electrons flow from the sample half-cell to the standard reference half-cell, and the sample-cell electrode is taken to be negative with respect to the standard-cell electrode. *The reduction potential of the X:X$^-$ couple is the observed voltage at the start of the experiment* (when X, X$^-$, and H$^+$ are 1 M). *The reduction potential of the H$^+$:H$_2$ couple is defined to be 0 volts.*

The meaning of the reduction potential is now evident. A negative reduction potential means that the reduced form of a substance has lower affinity for electrons than does H_2, as in the preceding example. A positive reduction potential means that the reduced form of a substance has higher affinity for electrons than does H_2. These comparisons refer to standard conditions—namely, 1 M oxidant, 1 M reductant, 1 M H^+, and 1 atmosphere H_2. Thus, *a strong reducing agent (such as NADH) is poised to donate electrons and has a negative reduction potential, whereas a strong oxidizing agent (such as O$_2$) is ready to accept electrons and has a positive reduction potential.*

The reduction potentials of many biologically important redox couples are known (Table 18.1). Table 18.1 is like those presented in chemistry texts except that a hydrogen ion concentration of 10^{-7} M (pH 7) instead of 1 M (pH 0) is the standard state adopted by biochemists. This difference is denoted by the prime in E'_0. Recall that the prime in $\Delta G^{\circ\prime}$ denotes a standard free-energy change at pH 7.

The standard free-energy change $\Delta G^{\circ\prime}$ is related to the change in reduction potential $\Delta E'_0$ by

$$\Delta G^{\circ\prime} = -nF\Delta E'_0$$

in which n is the number of electrons transferred, F is a proportionality constant called the *faraday* [23.06 kcal mol^{-1} V^{-1} (96.48 kJ mol^{-1} V^{-1})], $\Delta E'_0$ is in volts, and $\Delta G^{\circ\prime}$ is in kilocalories or kilojoules per mole.

The free-energy change of an oxidation-reduction reaction can be readily calculated from the reduction potentials of the reactants. For example, consider the reduction of pyruvate by NADH, catalyzed by lactate dehydrogenase.

$$\text{Pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{lactate} + \text{NAD}^+ \qquad \text{(a)}$$

The reduction potential of the NAD$^+$:NADH couple, or half-reaction, is -0.32 V, whereas that of the pyruvate: lactate couple is -0.19 V. By convention, reduction potentials (as in Table 18.1) refer to partial reactions written as reductions: oxidant + e$^-$ ⟶ reductant. Hence,

$$\text{Pyruvate} + 2H^+ + 2e^- \longrightarrow \text{lactate} \qquad E'_0 = -0.19\ \text{V} \qquad \text{(b)}$$

$$\text{NAD}^+ + H^+ + 2e^- \longrightarrow \text{NADH} \qquad E'_0 = -0.32\ \text{V} \qquad \text{(c)}$$

To obtain reaction a from reactions b and c, we need to reverse the direction of reaction c so that NADH appears on the left side of the arrow. In doing so, the sign of E'_0 must be changed.

$$\text{Pyruvate} + 2H^+ + 2e^- \longrightarrow \text{lactate} \qquad E'_0 = -0.19\ \text{V} \qquad \text{(b)}$$

$$NADH \longrightarrow NAD^+ + H^+ + 2\,e^- \qquad E'_0 = +0.32\,V \qquad (d)$$

For reaction b, the free energy can be calculated with $n = 2$.

$$\begin{aligned}\Delta G^{\circ\prime} &= -2 \times 23.06\ \text{kcal mol}^{-1}\,\text{V}^{-1} \times -0.19\,\text{V} \\ &= +8.8\ \text{kcal mol}^{-1}\ (36.7\ \text{kJ mol}^{-1})\end{aligned}$$

Likewise, for reaction d,

$$\begin{aligned}\Delta G^{\circ\prime} &= -2 \times 23.06\ \text{kcal mol}^{-1}\,\text{V}^{-1} \times +0.32\,\text{V} \\ &= -14.8\ \text{kcal mol}^{-1}\ (61.7\ \text{kJ mol}^{-1})\end{aligned}$$

Thus, the free energy for reaction a is given by

$$\begin{aligned}\Delta G^{\circ\prime} &= \Delta G^{\circ\prime}\ (\text{for reaction b}) + \Delta G^{\circ\prime}\ (\text{for reaction d}) \\ &= +8.8 + (-14.8) \\ &= -6.0\ \text{kcal mol}^{-1}\ (-25.1\ \text{kJ mol}^{-1})\end{aligned}$$

18.2.2. A 1.14-Volt Potential Difference Between NADH and O_2 Drives Electron Transport Through the Chain and Favors the Formation of a Proton Gradient

The driving force of oxidative phosphorylation is the electron transfer potential of NADH or $FADH_2$ relative to that of O_2. How much energy is released by the reduction of O_2 with NADH? Let us calculate $\Delta G^{\circ\prime}$ for this reaction. The pertinent half-reactions are

$$\tfrac{1}{2}\,O_2 + 2\,H^+ + 2\,e^- \longrightarrow H_2O \qquad E'_0 = +0.82\,V \qquad (a)$$

$$NAD^+ + H^+ + 2\,e^- \longrightarrow NADH \qquad E'_0 = -0.32\,V \qquad (b)$$

Subtracting reaction b from reaction a yields

$$\tfrac{1}{2}\,O_2 + NADH + H^+ \longrightarrow H_2O + NAD^+ \qquad (c)$$

The standard free energy for this reaction is then given by

$$\begin{aligned}\Delta G^{\circ\prime} &= -2 \times 23.06\ \text{kcal mol}^{-1}\,\text{V}^{-1} \times +0.82\,\text{V} - \\ &\qquad (-2 \times 23.06\ \text{kcal mol}^{-1}\,\text{V}^{-1} \times -0.32\,\text{V}) \\ &= -37.8\ \text{kcal mol}^{-1} - (14.8\ \text{kcal mol}^{-1}) \\ &= -52.6\ \text{kcal mol}^{-1}\ (-220.1\ \text{kJ mol}^{-1})\end{aligned}$$

This is a substantial release of free energy. Recall that $\Delta G^{\circ\prime}$ = - 7.5 kcal mol^{-1} (- 31.4 kJ mol^{-1}) for the hydrolysis of ATP. The released energy is used initially to generate a proton gradient that is then used for the synthesis of ATP and the transport of metabolites across the mitochondrial membrane.

How can the energy associated with a proton gradient be quantified? Recall that the free-energy change for a species moving from one side of a membrane where it is at concentration c_1 to the other side where it is at a concentration c_2 is given by

$$\Delta G = RT \ln (c_2/c_1) + ZF\Delta V = 2.303RT \log_{10} (c_2/c_1) + ZF\Delta V$$

in which Z is the electrical charge of the transported species and ΔV is the potential in volts across the membrane (Section 13.1.2). Under typical conditions for the inner mitochondrial membrane, the pH outside is 1.4 units lower than inside [corresponding to $\log_{10} (c_2/c_1)$ of 1.4] and the membrane potential is 0.14 V, the outside being positive. Because Z = +1 for protons, the free-energy change is (2.303 × 1.98 × 10^{-3} kcal mol^{-1} K^{-1} × 310 K × 1.4) + (+ 1 × 23.06 kcal mol^{-1} V^{-1} × 0.14 V) = 5.2 kcal mol^{-1} (21.8 kJ mol^{-1}). Thus, each proton that is transported out of the matrix to the cytosolic side corresponds to 5.2 kcal mol^{-1} of free energy.

18.2.3. Electrons Can Be Transferred Between Groups That Are Not in Contact

As will be discussed shortly, the electron-carrying groups in the protein constituents of the electron-transport chain are *flavins, iron-sulfur clusters, quinones, hemes,* and *copper ions.* How are electrons transferred between electron-carrying groups that are frequently buried in the interior of a protein in fixed positions and are therefore not directly in contact? Electrons can move through space, even through a vacuum. However, the rate of electron transfer through space falls off rapidly as the electron donor and electron acceptor move apart from each other, decreasing by a factor of 10 for each increase in separation of 0.8 Å. The protein environment provides more-efficient pathways for electron conduction: typically, the rate of electron transfer decreases by a factor of 10 every 1.7 Å (Figure 18.7). For groups in contact, electron-transfer reactions can be quite fast with rates of approximately 10^{13} s^{-1}. Within proteins in the electron-transport chain, electron-carrying groups are typically separated by 15 Å beyond their van der Waals contact distance. For such separations, we expect electron-transfer rates of approximately 10^4 s^{-1} (i.e., electron transfer in less than 1 ms), assuming that all other factors are optimal. Without the mediation of the protein, an electron transfer over this distance would take approximately 1 day.

Another important factor in determining the rate of electron transfer is the driving force, the free-energy change associated with the reaction (Figure 18.8). Like the rates of most reactions, those of electron-transfer reactions tend to increase as the free-energy change for the reaction becomes more favorable. Interestingly, however, each electron-transfer reaction has an optimal driving force; making the reaction more favorable beyond this point decreases the rate of the electron-transfer process. This so-called *inverted region* is of tremendous importance for the light reactions of photosynthesis, to be discussed in Chapter 19. For the purposes of the electron-transport chain, the effects of distance and driving force combine to determine which pathway, among the set of those possible, will be used at each stage in the course of a reaction.

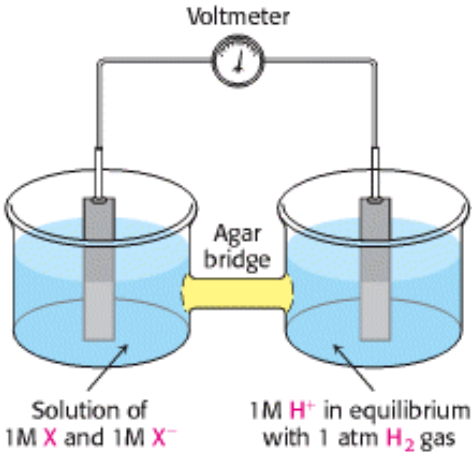


Figure 18.6. Measurement of Redox Potential. Apparatus for the measurement of the standard oxidation-reduction potential of a redox couple. Electrons, but not X or X^-, can flow through the agar bridge.

Table 18.1. Standard reduction potentials of some reactions

Oxidant	Reductant	*n*	E'_0 (V)
Succinate + CO_2	α-Ketoglutarate	2	- 0.67
Acetate	Acetaldehyde	2	- 0.60
Ferredoxin (oxidized)	Ferredoxin (reduced)	1	- 0.43
2 H^+	H_2	2	- 0.42
NAD^+	NADH + H^+	2	- 0.32
$NADP^+$	NADPH + H^+	2	- 0.32
Lipoate (oxidized)	Lipoate (reduced)	2	- 0.29
Glutathione (oxidized)	Glutathione (reduced)	2	- 0.23
FAD	$FADH_2$	2	- 0.22
Acetaldehyde	Ethanol	2	- 0.20
Pyruvate	Lactate	2	- 0.19
Fumarate	Succinate	2	0.03
Cytochrome *b* (+3)	Cytochrome *b* (+2)	1	0.07
Dehydroascorbate	Ascorbate	2	0.08
Ubiquinone (oxidized)	Ubiquinone (reduced)	2	0.10
Cytochrome *c* (+3)	Cytochrome *c* (+2)	1	0.22
Fe (+3)	Fe (+2)	1	0.77
$^1/_2$ O_2+ 2 H^+	H_2O	2	0.82

Note: E'_0 is the standard oxidation-reduction potential (pH 7, 25°C) and *n* is the number of electrons transferred. E'_0 refers to the partial reaction written as

$$\text{Oxidant} + e^- \longrightarrow \text{reductant}$$

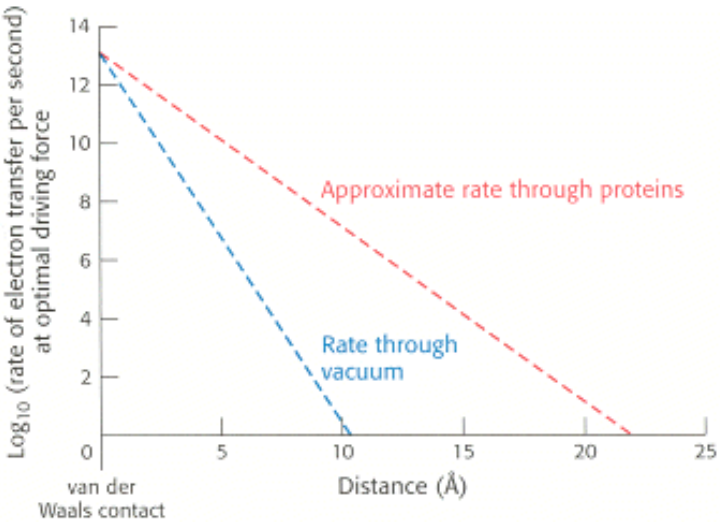


Figure 18.7. Distance Dependence of Electron-Transfer Rate. The rate of electron transfer decreases as the electron donor and the electron acceptor move apart. In a vacuum, the rate decreases by a factor of 10 for every increase of 0.8 Å. In proteins, the rate decreases more gradually, by a factor of 10 for every increase of 1.7 Å. This rate is only approximate because variations in the structure of the intervening protein medium can affect the rate.

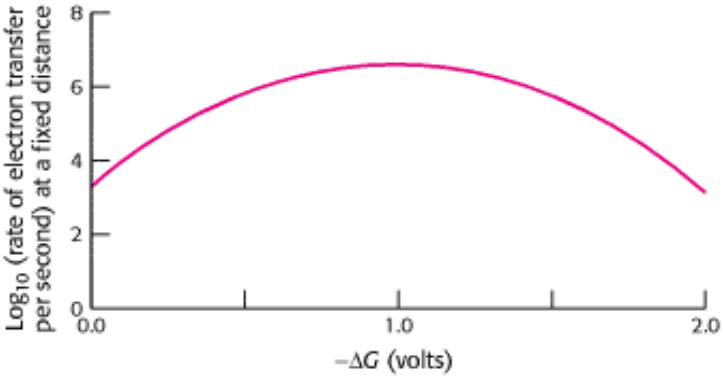


Figure 18.8. Free-Energy Dependence of Electron-Transfer Rate. The rate of an electron-transfer reaction at first increases as the driving force for the reaction increases. The rate reaches a maximum and then decreases at very large driving forces.

18.3. The Respiratory Chain Consists of Four Complexes: Three Proton Pumps and a Physical Link to the Citric Acid Cycle

Electrons are transferred from NADH to O_2 through a chain of three large protein complexes called *NADH-Q oxidoreductase*, *Q-cytochrome* c *oxido-reductase*, and *cytochrome* c *oxidase* (Figure 18.9 and Table 18.2). *Electron flow within these transmembrane complexes leads to the transport of protons across the inner mitochondrial membrane.* Electrons are carried from NADH-Q oxidoreductase to Q-cytochrome *c* oxidoreductase, the second complex of the chain, by the reduced form of *coenzyme Q (Q)*, also known as *ubiquinone* because it is a *ubi*quitous *quinone* in biological systems. Ubiquinone is a hydrophobic quinone that diffuses rapidly within the inner mitochondrial membrane. Ubiquinone also carries electrons from $FADH_2$, generated in succinate dehydrogenase in the citric acid cycle, to Q-cytochrome *c* oxidoreductase, generated through *succinate-Q reductase.* Cytochrome *c*, a small, soluble protein, shuttles electrons from Q-cytochrome *c* oxidoreductase to cytochrome *c* oxidase, the final component in the chain and the one that catalyzes the reduction of O_2. NADH-Q oxidoreductase, succinate-Q reductase, Q-cytochrome *c* oxidoreductase,

and cytochrome *c* oxidase are also called *Complex I, II, III,* and *IV,* respectively. Succinate-Q reductase (Complex II), in contrast with the other complexes, does not pump protons.

Coenzyme Q is a quinone derivative with a long isoprenoid tail. The number of five-carbon isoprene units in coenzyme Q depends on the species. The most common form in mammals contains 10 isoprene units (coenzyme Q_{10}). For simplicity, the subscript will be omitted from this abbreviation because all varieties function in an identical manner. Quinones can exist in three oxidation states (Figure 18.10). In the fully oxidized state (Q), coenzyme Q has two keto groups. The addition of one electron and one proton results in the semiquinone form (QH·). The semiquinone form is relatively easily deprotonated to form a semiquinone radical anion ($Q\cdot^-$). The addition of a second electron and proton generates ubiquinol (QH_2), the fully reduced form of coenzyme Q, which holds its protons more tightly. Thus, *for quinones, electron-transfer reactions are coupled to proton binding and release,* a property that is key to transmembrane proton transport.

18.3.1. The High-Potential Electrons of NADH Enter the Respiratory Chain at NADH-Q Oxidoreductase

The electrons of NADH enter the chain at *NADH-Q oxidoreductase* (also called *NADH dehydrogenase*), an enormous enzyme (880 kd) consisting of at least 34 polypeptide chains. The construction of this proton pump, like that of the other two in the respiratory chain, is a cooperative effort of genes residing in both the mitochondria and the nucleus. The structure of this enzyme has been determined only at moderate resolution (Figure 18.11). NADH-Q oxidoreductase is L-shaped, with a horizontal arm lying in the membrane and a vertical arm that projects into the matrix. Although a detailed understanding of the mechanism is likely to require higher-resolution structural information, some aspects of the mechanism have been established.

The reaction catalyzed by this enzyme appears to be

$$NADH + Q + 5\,H^+_{matrix} \longrightarrow NAD^+ + QH_2 + 4\,H^+_{cytosol}$$

The initial step is the binding of NADH and the transfer of its two high-potential electrons to the *flavin mononucleotide (FMN)* prosthetic group of this complex to give the reduced form, $FMNH_2$. Like quinones, flavins bind protons when they are reduced. FMN can also accept one electron instead of two (or $FMNH_2$ can donate one electron) by forming a semiquinone radical intermediate (Figure 18.12). The electron acceptor of FMN, the isoalloxazine ring, is identical with that of FAD. Electrons are then transferred from $FMNH_2$ to a series of *iron-sulfur clusters,* the second type of prosthetic group in NADH-Q oxidoreductase.

Fe-S clusters in *iron-sulfur proteins* (also called *nonheme iron proteins*) play a critical role in a wide range of reduction reactions in biological systems. Several types of Fe-S clusters are known (Figure 18.13). In the simplest kind, a single iron ion is tetrahedrally coordinated to the sulfhydryl groups of four cysteine residues of the protein. A second kind, denoted by 2Fe-2S, contains two iron ions and two inorganic sulfides. Such clusters are usually coordinated by four cysteine residues, although exceptions exist, as we shall see when we consider Q-cytochrome *c* oxidoreductase. A third type, designated 4Fe-4S, contains four iron ions, four inorganic sulfides, and four cysteine residues. We encountered a variation of this type of cluster in aconitase in Section 17.1.4. NADH-Q oxidoreductase contains both 2Fe-2S and 4Fe-4S clusters. Iron ions in these Fe-S complexes cycle between Fe^{2+} (reduced) or Fe^{3+}(oxidized) states. Unlike quinones and flavins, iron-sulfur clusters generally undergo oxidation-reduction reactions without releasing or binding protons.

Electrons in the iron-sulfur clusters of NADH-Q oxidoreductase are shuttled to coenzyme Q. *The flow of two electrons from NADH to coenzyme Q through NADH-Q oxidoreductase leads to the pumping of four hydrogen ions out of the matrix of the mitochondrion.* The details of this process remain the subject of active investigation. However, the coupled electron- proton transfer reactions of Q are crucial. NADH binds to a site on the vertical arm and transfers its electrons to FMN. These electrons flow within the vertical unit to three 4Fe-4S centers and then to a bound Q. *The reduction of Q to*

QH_2 results in the uptake of two protons from the matrix (Figure 18.14). The pair of electrons on bound QH_2 are transferred to a 4Fe-4S center and the protons are released on the cytosolic side. Finally, these elections are transferred to a *mobile Q* in the hydrophobic core of the membrane, resulting in the uptake of two additional protons from the matrix. The challenge is to delineate the binding events and conformational changes induced by these electron transfers and learn how the uptake and release of protons from the appropriate sides of the membrane is facilitated.

18.3.2. Ubiquinol Is the Entry Point for Electrons from $FADH_2$ of Flavoproteins

The citric acid cycle enzyme succinate dehydrogenase, which generates $FADH_2$ with the oxidation of succinate to fumarate (Section 17.1.8), is part of the *succinate-Q reductase complex (Complex II)*, an integral membrane protein of the inner mitochondrial membrane. $FADH_2$ does not leave the complex. Rather, its electrons are transferred to Fe-S centers and then to Q for entry into the electron-transport chain. Two other enzymes that we will encounter later, *glycerol phosphate dehydrogenase* (Section 18.5.1) and *fatty acyl CoA dehydrogenase* (Section 22.2.4), likewise transfer their highpotential electrons from $FADH_2$ to Q to form ubiquinol (QH_2), the reduced state of ubiquinone. The succinate-Q reductase complex and other enzymes that transfer electrons from $FADH_2$ to Q, in contrast with NADH-Q oxidoreductase, do not transport protons. Consequently, less ATP is formed from the oxidation of $FADH_2$ than from NADH.

18.3.3. Electrons Flow from Ubiquinol to Cytochrome *c* Through Q-Cytochrome *c* Oxidoreductase

The second of the three proton pumps in the respiratory chain is *Q-cytochrome* c *oxidoreductase* (also known as *Complex III* and cytochrome reductase). A cytochrome is an electron-transferring protein that contains a heme prosthetic group. The iron ion of a cytochrome alternates between a reduced ferrous (+2) state and an oxidized ferric (+3) state during electron transport. The function of Q-cytochrome *c* oxidoreductase is to catalyze the transfer of electrons from QH_2 to oxidized *cytochrome* c *(cyt c)*, a water-soluble protein, and concomitantly pump protons out of the mitochondrial matrix.

$$QH_2 + 2\,\text{Cyt}\,c_{ox} + 2\,H^+_{matrix} \longrightarrow Q + 2\,\text{Cyt}\,c_{red} + 4\,H^+_{cytosol}$$

Q-cytochrome *c* oxidoreductase is a dimer with each monomer containing 11 subunits (Figure 18.15). Q-cytochrome *c* oxidoreductase itself contains a total of three hemes, contained within two cytochrome subunits: two *b*-type hemes, termed heme b_L (L for low affinity) and heme b_H (H for high affinity), within cytochrome *b*, and one *c*-type heme within cytochrome c_1. The prosthetic group of the heme in cytochromes *b*, c_1, and *c* is iron- protoporphyrin IX, the same heme as in myoglobin and hemoglobin (Section 10.2.1). The hemes in cytochromes *c* and c_1, in contrast with those in cytochrome *b*, are covalently attached to the protein (Figure 18.16). The linkages are thioethers formed by the addition of the sulfhydryl groups of two cysteine residues to the vinyl groups of the heme. Because of these groups, this enzyme is also known as cytochrome bc_1. In addition to the hemes, the enzyme also contains an iron-sulfur protein with an 2Fe-2S center. This center, termed the *Rieske center*, is unusual in that one of the iron ions is coordinated by two histidine residues rather than two cysteine residues. This coordination stabilizes the center in its reduced form, raising its reduction potential. Finally, Q-cytochrome *c* oxidoreductase contains two distinct binding sites for ubiquinone termed Q_o and Q_i, with the Q_i site lying closer to the inside of the matrix.

18.3.4. Transmembrane Proton Transport: The Q Cycle

The mechanism for the coupling of electron transfer from Q to cytochrome *c* to transmembrane proton transport is known as the *Q cycle* (Figure 18.17). The Q cycle also facilitates the switch from the two-electron carrier ubiquinol to the one-electron carrier cytochrome *c*. The cycle begins as ubiquinol (QH_2) binds in the Q_o site. Ubiquinol transfers its

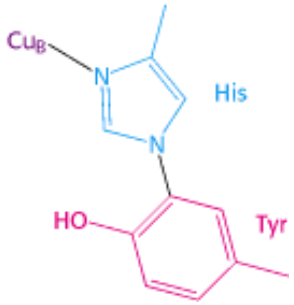
electrons, one at a time. One electron flows first to the Rieske 2Fe-2S cluster, then to cytochrome c_1, and finally to a molecule of oxidized cytochrome *c*, converting it into its reduced form. The reduced cytochrome *c* molecule is free to diffuse away from the enzyme. The second electron is transferred first to cytochrome b_L, then to cytochrome b_H, and finally to an oxidized uniquinone bound in the Q_i site. This quinone (Q) molecule is reduced to a semiquinone anion ($Q \cdot^-$). Importantly, as the QH_2 in the Q_o site is oxidized to Q, its protons are released to the cytosolic side of the membrane. This Q molecule in the Q_o site is free to diffuse out into the ubiquinone pool.

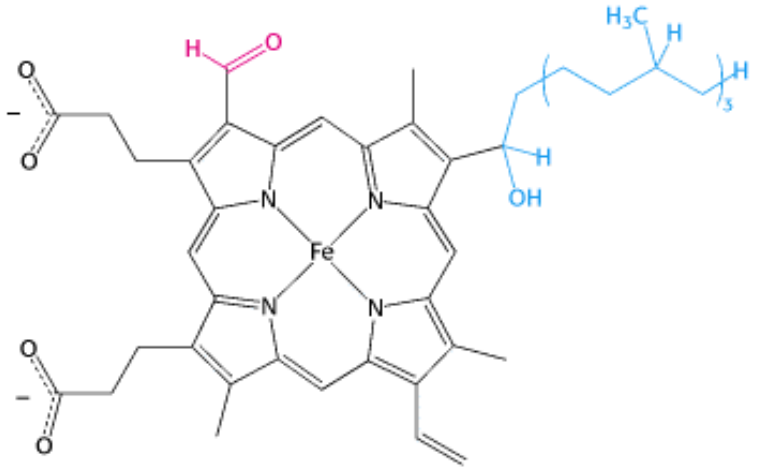
At this point, $Q \cdot^-$ resides in the Q_i site. A second molecule of QH_2 binds to the Q_o site and reacts in the same way as the first. One of its electrons is transferred through the Rieske center and cytochrome c_1 to reduce a second molecule of cytochrome *c*. The other electron goes through cytochromes b_L and b_H to $Q \cdot^-$ bound in the Q_i site. On the addition of the second electron, this quinone radical anion takes up two protons from the matrix side to form QH_2. *The removal of these two protons from the matrix contributes to the formation of the proton gradient.* At the end of the Q cycle, two molecules of QH_2 are oxidized to form two molecules of Q, and one molecule of Q is reduced to QH_2, two molecules of cytochrome *c* are reduced, four protons are released on the cytoplasmic side, and two protons are removed from the mitochondrial matrix.

18.3.5. Cytochrome *c* Oxidase Catalyzes the Reduction of Molecular Oxygen to Water

The final stage of the electron-transport chain is the oxidation of the reduced cytochrome *c* generated by Complex III, which is coupled to the reduction of O_2 to two molecules of H_2O. This reaction is catalyzed by *cytochrome* c *oxidase (Complex IV)*. The four-electron reduction of oxygen directly to water without the release of intermediates poses a challenge. Nevertheless, this reaction is quite thermodynamically favorable. From the reduction potentials in Table 18.1, the standard free-energy change for this reaction is calculated to be $\Delta G^{\circ\prime} = -55.4$ kcal mol^{-1} (-231.8 kJ mol^{-1}). As much of this free energy as possible must be captured in the form of a proton gradient for subsequent use in ATP synthesis.

Bovine cytochrome *c* oxidase is reasonably well understood at the structural level (Figure 18.18). It consists of 13 subunits, of which 3 (called *subunits I, II,* and *III*) are encoded by the mitochondrial genome. Cytochrome *c* oxidase contains two heme A groups and three copper ions, arranged as two copper centers, designated A and B. One center, Cu_A/Cu_A, contains two copper ions linked by two bridging cysteine residues. This center initially accepts electrons from reduced cytochrome *c*. The remaining copper ion, Cu_B, is coordinated by three histidine residues, one of which is modified by covalent linkage to a tyrosine residue. Heme A differs from the heme in cytochrome *c* and c_1 in three ways: (1) a formyl group replaces a methyl group, (2) a C_{15} hydrocarbon chain replaces one of the vinyl groups, and (3) the heme is not covalently attached to the protein.





Heme A

The two heme A molecules, termed *heme* a and *heme* a_3, have distinct properties because they are located in different environments within cytochrome *c* oxidase. Heme *a* functions to carry electrons from Cu_A/Cu_A, whereas heme a_3 passes electrons to Cu_B, to which it is directly adjacent. Together, *heme* a_3 *and* Cu_B *form the active center at which* O_2 *is reduced to* H_2O.

The catalytic cycle begins with the enzyme in its fully oxidized form (Figure 18.19). One molecule of reduced cytochrome *c* transfers an electron, initially to Cu_A/Cu_A. From there, the electron moves to heme *a*, then to heme a_3, and finally to Cu_B, which is reduced from the Cu^{2+} (cupric) form to the Cu^+ (cuprous) form. A second molecule of cytochrome *c* introduces a second electron that flows down the same path, stopping at heme a_3, which is reduced to the Fe^{2+} form. Recall that the iron in hemoglobin is in the Fe^{2+} form when it binds oxygen (Section 10.2.1). Thus, at this stage, cytochrome *c* oxidase is poised to bind oxygen and does so. The proximity of Cu_B in its reduced form to the heme a_3-oxygen complex allows the oxygen to be reduced to peroxide (O_2^{2-}), which forms a bridge between the Fe^{3+} in heme a_3 and Cu_B^{2+} (Figure 18.20). The addition of a third electron from cytochrome *c* as well as a proton results in the cleavage of the O-O bond, yielding a ferryl group, $Fe^{4+}=O$, at heme a_3 and Cu_B^{2+}-OH. The addition of the final electron from cytochrome *c* and a second proton reduces the ferryl group to Fe^{3+}-OH. Reaction with two additional protons allows the release of two molecules of water and resets the enzyme to its initial, fully oxidized form.

This reaction can be summarized as

$$4\,\text{Cyt}\,c_{\text{red}} + 4\,H^+_{\text{matrix}} + O_2 \longrightarrow 4\,\text{Cyt}\,c_{\text{ox}} + 2\,H_2O$$

The four protons in this reaction come exclusively from the matrix. Thus, the consumption of these four protons contributes directly to the proton gradient. Recall that each proton contributes 5.2 kcal mol^{-1} (21.8 kJ mol^{-1}) to the free energy associated with the proton gradient; so these four protons contribute 20.8 kcal mol^{-1} (87.2 kJ mol^{-1}), an amount substantially less than the free energy available from the reduction of oxygen to water. Remarkably, *cytochrome* c *oxidase evolved to pump four additional protons from the matrix to the cytoplasmic side of the membrane in the course of each reaction cycle for a total of eight protons removed from the matrix* (Figure 18.21). The details of how these protons are transported through the protein is still under study. However, two effects contribute to the mechanism. First, charge neutrality tends to be maintained in the interior of proteins. Thus, the addition of an electron to a site inside a protein tends to favor the binding of a proton to a nearby site. Second, conformational changes take place, particularly around the heme a_3-Cu_B center, in the course of the reaction cycle, and these changes must be used to allow protons to enter the protein exclusively from the matrix side and to exit exclusively to the cytosolic side. Thus, the overall process catalyzed by cytochrome c oxidase is

$$4\,\text{Cyt}\,c_{red} + 8\,H^+_{matrix} + O_2 \longrightarrow 4\,\text{Cyt}\,c_{ox} + 2\,H_2O + 4\,H^+_{cytosol}$$

As discussed earlier, molecular oxygen is an ideal terminal electron acceptor, because its high affinity for electrons provides a large thermodynamic driving force. However, *danger lurks in the reduction of* O_2. The transfer of four electrons leads to safe products (two molecules of H_2O), but partial reduction generates hazardous compounds. In particular, *the transfer of a single electron to* O_2 *forms superoxide anion, whereas the transfer of two electrons yields peroxide.*

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow{e^-} O_2^{2-}$$

Superoxide anion — Peroxide

These compounds and, particularly, their reaction products can be quite harmful to a variety of cell components. The strategy for the safe reduction of O_2 is clear from the discussion of the reaction cycle: *the catalyst does not release partly reduced intermediates.* Cytochrome *c* oxidase meets this crucial criterion by holding O_2 tightly between Fe and Cu ions.

18.3.6. Toxic Derivatives of Molecular Oxygen Such as Superoxide Radical Are Scavenged by Protective Enzymes

Although cytochrome *c* oxidase and other proteins that reduce O_2 are remarkably successful in not releasing intermediates, small amounts of superoxide anion and hydrogen peroxide are unavoidably formed. Superoxide, hydrogen peroxide, and species that can be generated from them such as OH· are collectively referred to as *reactive oxygen species* or *ROS.*

What are the cellular defense strategies against oxidative damage by ROS? Chief among them is the enzyme *superoxide dismutase.* This enzyme scavenges superoxide radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen.

$$2\,O_2^{\cdot-} + 2\,H^+ \xrightleftharpoons{\text{Superoxide dismutase}} O_2 + H_2O_2$$

Dismutation

A reaction in which a single reactant is converted into two different products.

Eukaryotes contain two forms of this enzyme, a manganese-containing version located in mitochondria and a copper-zinc-dependent cytosolic form. These enzymes perform the dismutation reaction by a similar mechanism (Figure 18.22). The oxidized form of the enzyme is reduced by superoxide to form oxygen. The reduced form of the enzyme, formed in this reaction, then reacts with a second superoxide ion to form peroxide, which takes up two protons along the reaction path to yield hydrogen peroxide.

The hydrogen peroxide formed by superoxide dismutase and by other processes is scavenged by *catalase,* a ubiquitous heme protein that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen.

$$2\,H_2O_2 \xrightleftharpoons{\text{Catalase}} O_2 + 2\,H_2O$$

Superoxide dismutase and catalase are remarkably efficient, performing their reactions at or near the diffusion-limited rate (Section 8.4.2). Other cellular defenses against oxidative damage include the antioxidant vitamins, vitamins E and C. Because it is lipophilic, vitamin E is especially useful in protecting membranes from lipid peroxidation.

The importance of the cell's defense against ROS is demonstrated by the presence of superoxide dismutase in all aerobic organisms. *Escherichia coli* mutants lacking this enzyme are highly vulnerable to oxidative damage. Moreover, oxidative damage is believed to cause, at least in part, a growing number of diseases (Table 18.3).

18.3.7. The Conformation of Cytochrome *c* Has Remained Essentially Constant for More Than a Billion Years

Cytochrome c is present in all organisms having mitochondrial respiratory chains: plants, animals, and eukaryotic microorganisms. This electron carrier evolved more than 1.5 billion years ago, before the divergence of plants and animals. Its function has been conserved throughout this period, as evidenced by the fact that *the cytochrome* c *of any eukaryotic species reacts in vitro with the cytochrome* c *oxidase of any other species tested thus far.* Finally, some prokaryotic cytochromes, such as cytochrome *c2* from a photosynthetic bacterium and cytochrome *c* 550 from a denitrifying bacterium, closely resemble cytochrome *c* from tuna heart mitochondria (Figure 18.23). This evidence attests that the structural and functional characteristics of cytochrome *c* present an efficient evolutionary solution to electron transfer.

The resemblance among cytochrome *c* molecules extends to the level of amino acid sequence. Because of the molecule's relatively small size and ubiquity, the amino acid sequences of cytochrome *c* from more than 80 widely ranging eukaryotic species were determined by direct protein sequencing by Emil Smith, Emanuel Margoliash, and others. Comparison of these sequences revealed that *26 of 104 residues have been invariant for more than one and a half billion years of evolution.* A phylogenetic tree, constructed from the amino acid sequences of cytochrome *c*, reveals the evolutionary relationships between many animal species (Figure 18.24).

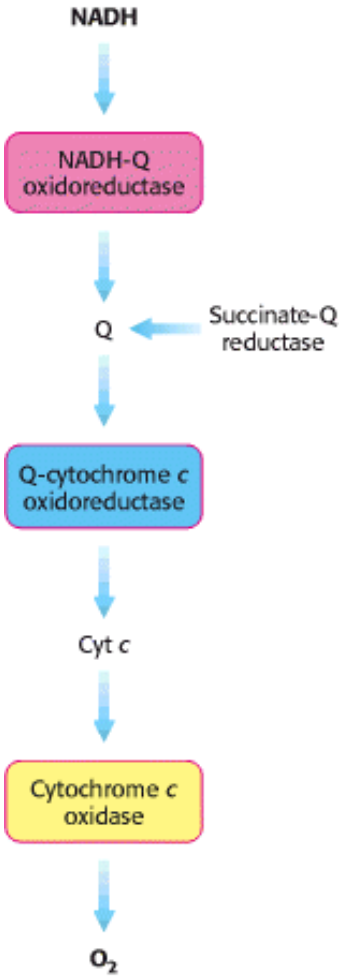


Figure 18.9. Sequence of Electron Carriers in the Respiratory Chain.

Table 18.2. Components of the mitochondrial electron-transport chain

				Oxidant or reductant		
Enzyme complex	Mass (kd)	Subunits	Prosthetic group	Matrix side	Membrane core	Cytosolic side
NADH-Q oxidoreductase	880	≧ 34	FMN	NADH	Q	
			Fe-S			
Succinate-Q reductase	140	4	FAD	Succinate	Q	
			Fe-S			
Q-cytochrome *c* oxidoreductase	250	10	Heme b_H		Q	Cytochrome *c*
			Heme b_L			
			Heme c_1			
			Fe-S			
Cytochrome *c* oxidase	160	10	Heme *a*			Cytochrome *c*

Heme a_3	
Cu_A and Cu_B	

Sources: J. W. DePierre and L. Ernster, *Annu. Rev. Biochem.* 46(1977):215; Y. Hatefi, *Annu Rev. Biochem.* 54(1985);1015; and J. E. Walker, *Q. Rev. Biophys.* 25(1992):253.

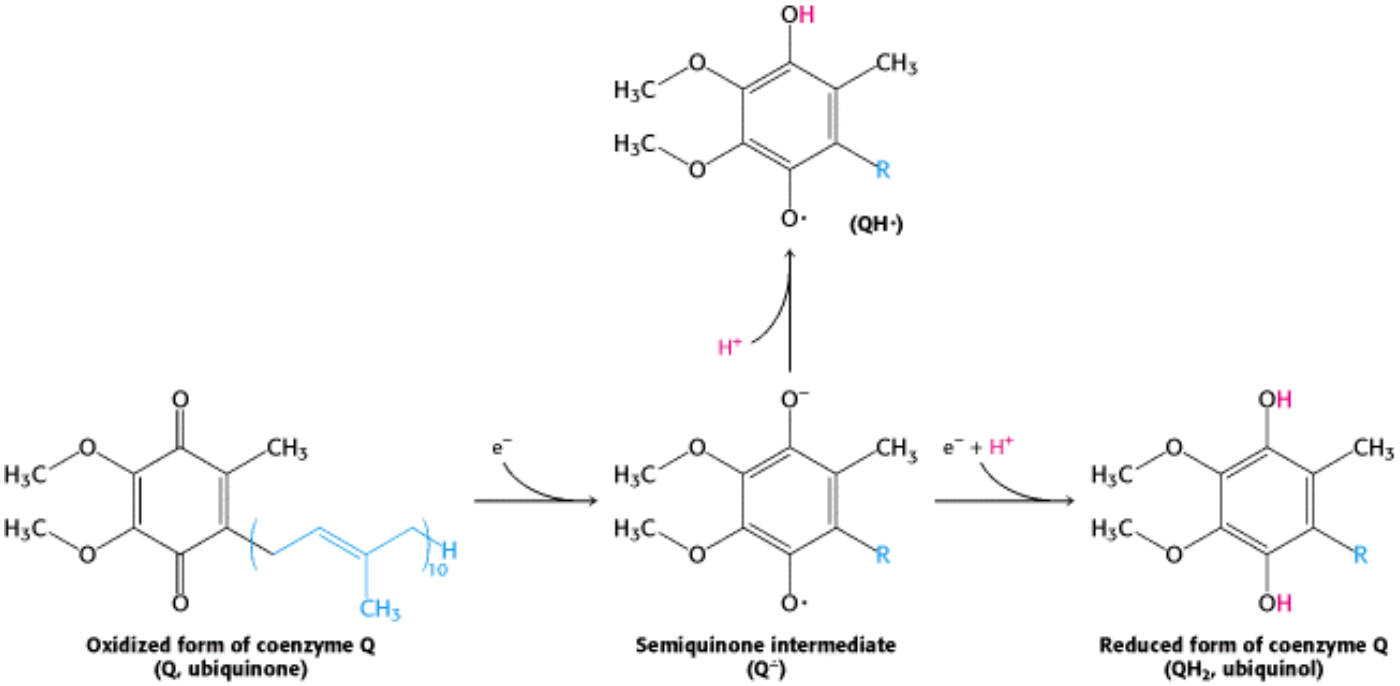


Figure 18.10. Oxidation States of Quinones. The reduction of ubiquinone (Q) to ubiquinol (QH_2) proceeds through a semiquinone anion intermediate ($Q^{\bullet -}$).

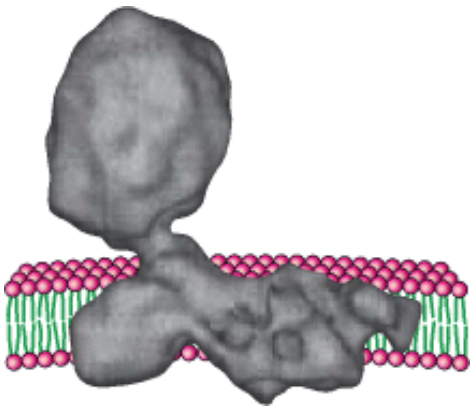


Figure 18.11. Structure of NADH-Q Oxidoreductase (Complex I). The structure, determined by electron microscopy at 22-Å resolution, consists of a membrane-spanning part and a long arm that extends into the matrix. NADH is oxidized in the arm, and the electrons are transferred to reduce Q in the membrane. [After N. Grigorieff, *J. Mol. Biol.* 277 (1998):1033−1048.]

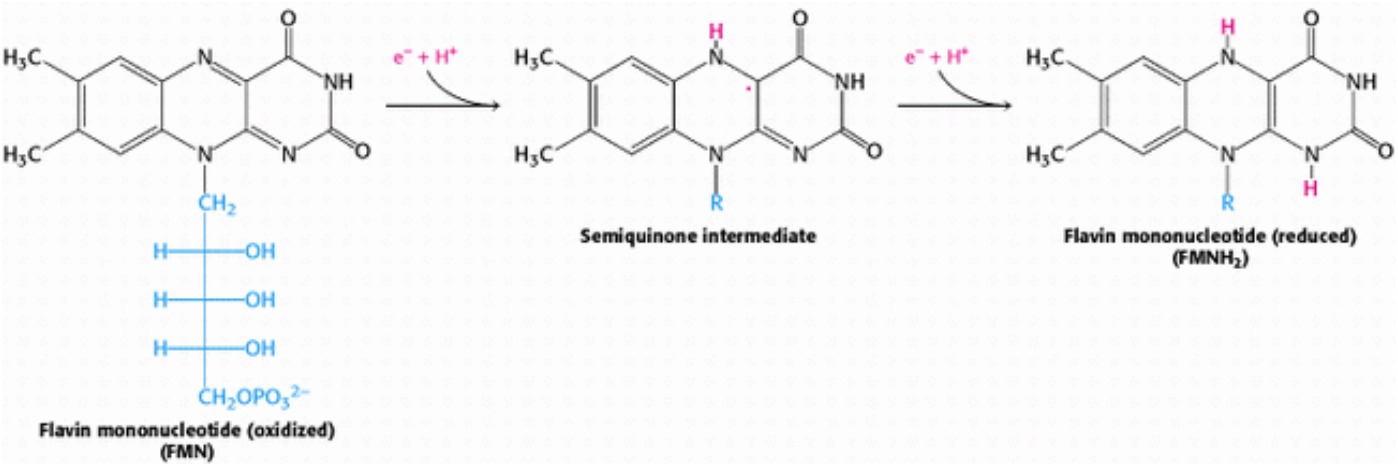


Figure 18.12. Oxidation States of Flavins. The reduction of flavin mononucleotide (FMN) to $FMNH_2$ proceeds through a semiquinone intermediate.

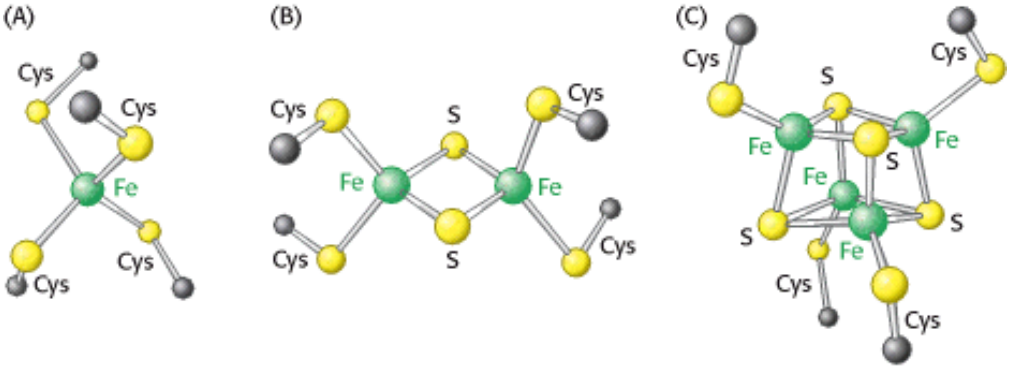


Figure 18.13. Iron-Sulfur Clusters. (A) A single iron ion bound by four cysteine residues. (B) 2Fe-2S cluster with iron ions bridged by sulfide ions. (C) 4Fe-4S cluster. Each of these clusters can undergo oxidation-reduction reactions.

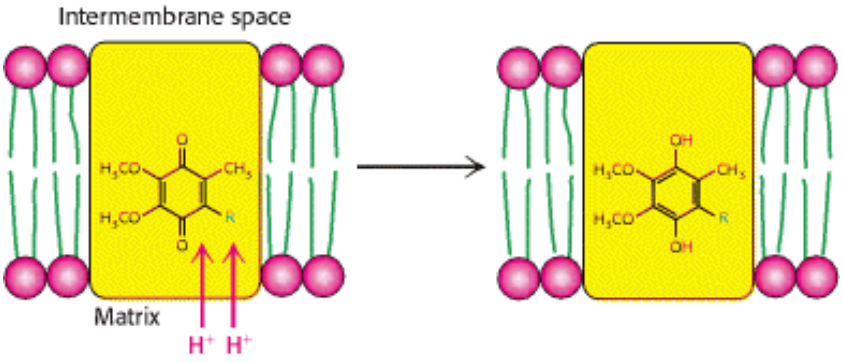


Figure 18.14. Coupled Electron-Proton Transfer Reactions. The reduction of a quinone (Q) to QH_2 in an appropriate site can result in the uptake of two protons from the mitochondrial matrix.

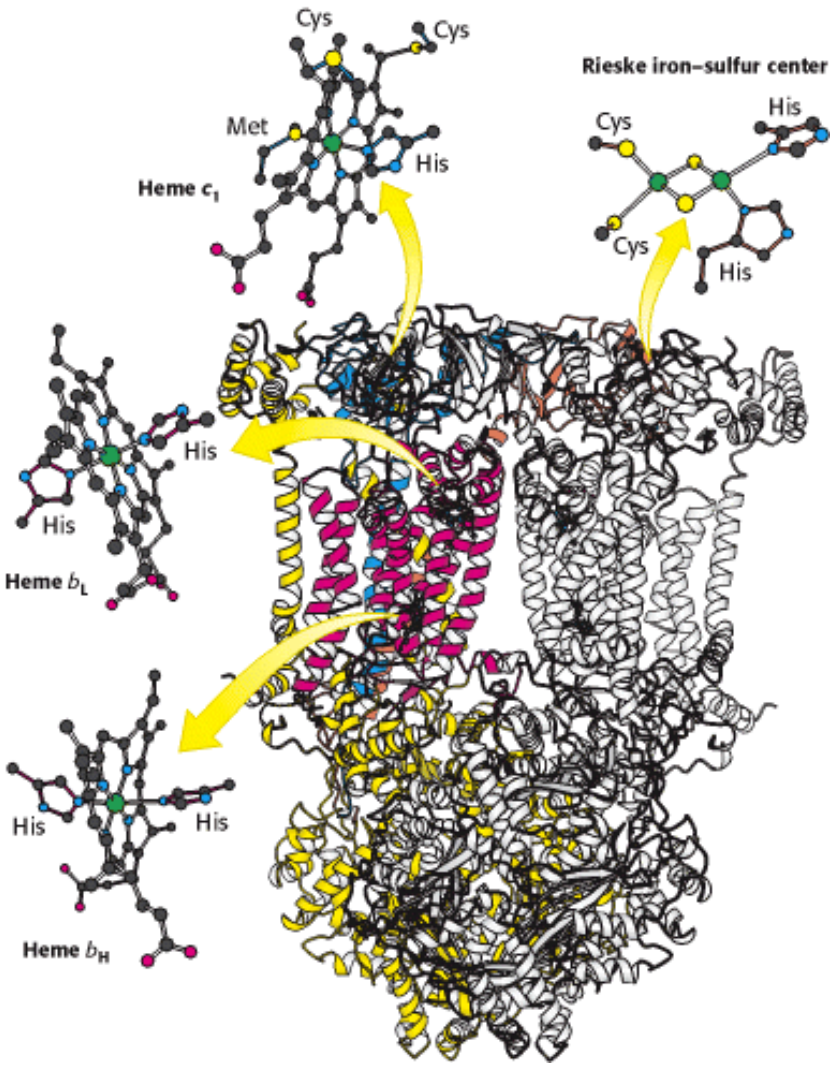


Figure 18.15. Structure of Q-Cytochrome *C* Oxidoreductase (Cytochrome *BC* $_1$). This enzyme is a homodimer with 11 distinct polypeptide chains. The major prosthetic groups, three hemes and a 2Fe-2S cluster, mediate the electron-transfer reactions between quinones in the membrane and cytochrome *c* in the intermembrane space.

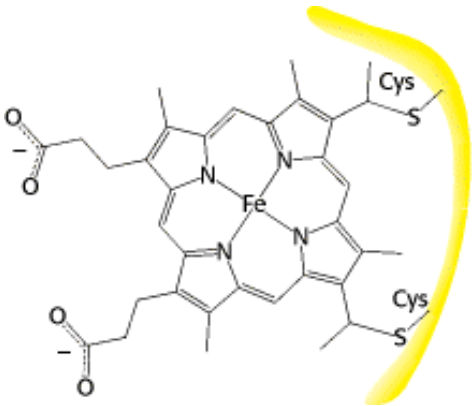


Figure 18.16. Attachment of *C* -Type Cytochromes. A heme group is covalently attached to a protein through thioether linkages formed by the addition of sulfhydryl groups of cysteine residues to vinyl groups on protoporphyrin.

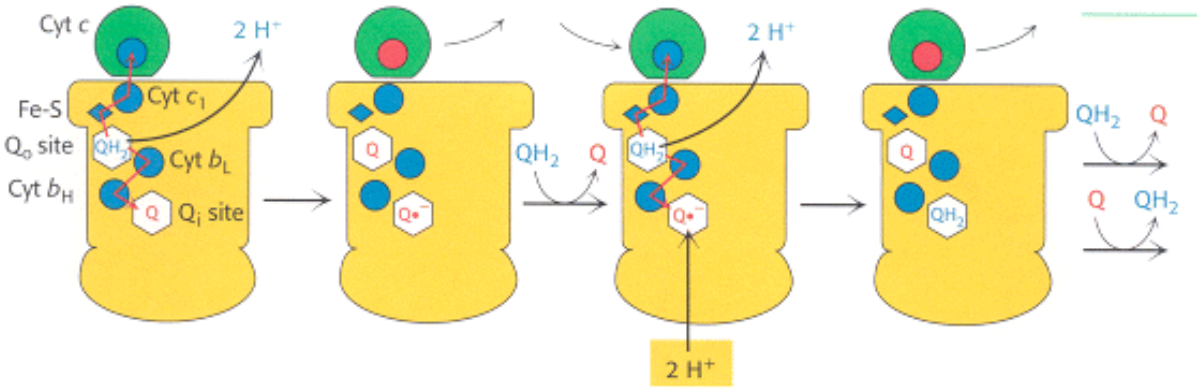


Figure 18.17. Q Cycle. The two electrons of a bound QH_2 are transferred, one to cytochrome *c* and the other to a bound Q to form the semiquinone $Q^{\bullet -}$. The newly formed Q dissociates and is replaced by a second QH_2, which also gives up its electrons, one to a second molecule of cytochrome *c* and the other to reduce $Q^{\bullet -}$ to QH_2. This second electron transfer results in the uptake of two protons from the matrix. Prosthetic groups are shown in their oxidized forms in blue and in their reduced forms in red.

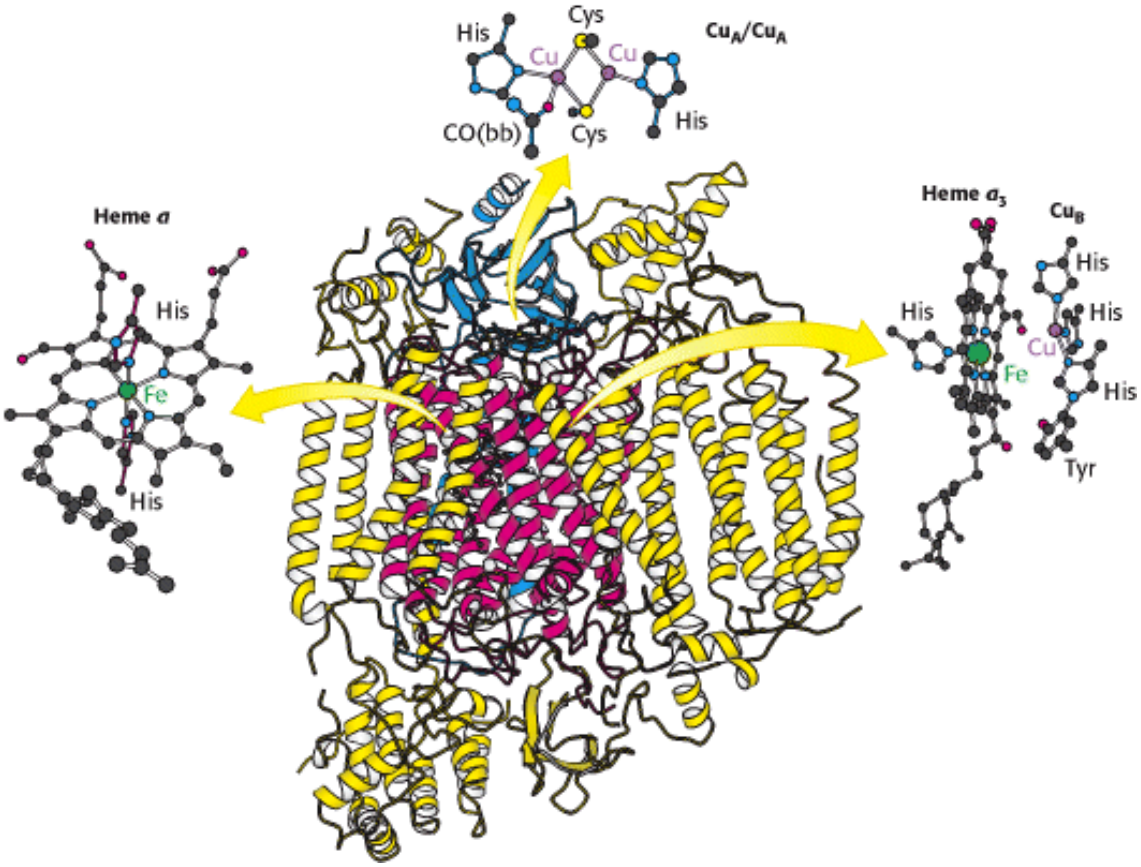


Figure 18.18. Structure of Cytochrome *C* Oxidase. This enzyme consists of 13 polypeptide chains. The major prosthetic groups include Cu_A/Cu_A, heme *a*, and heme a_3-Cu_B. Heme a_3-Cu_B is the site of the reduction of oxygen to water. CO(bb) is a carbonyl group of the peptide backbone.

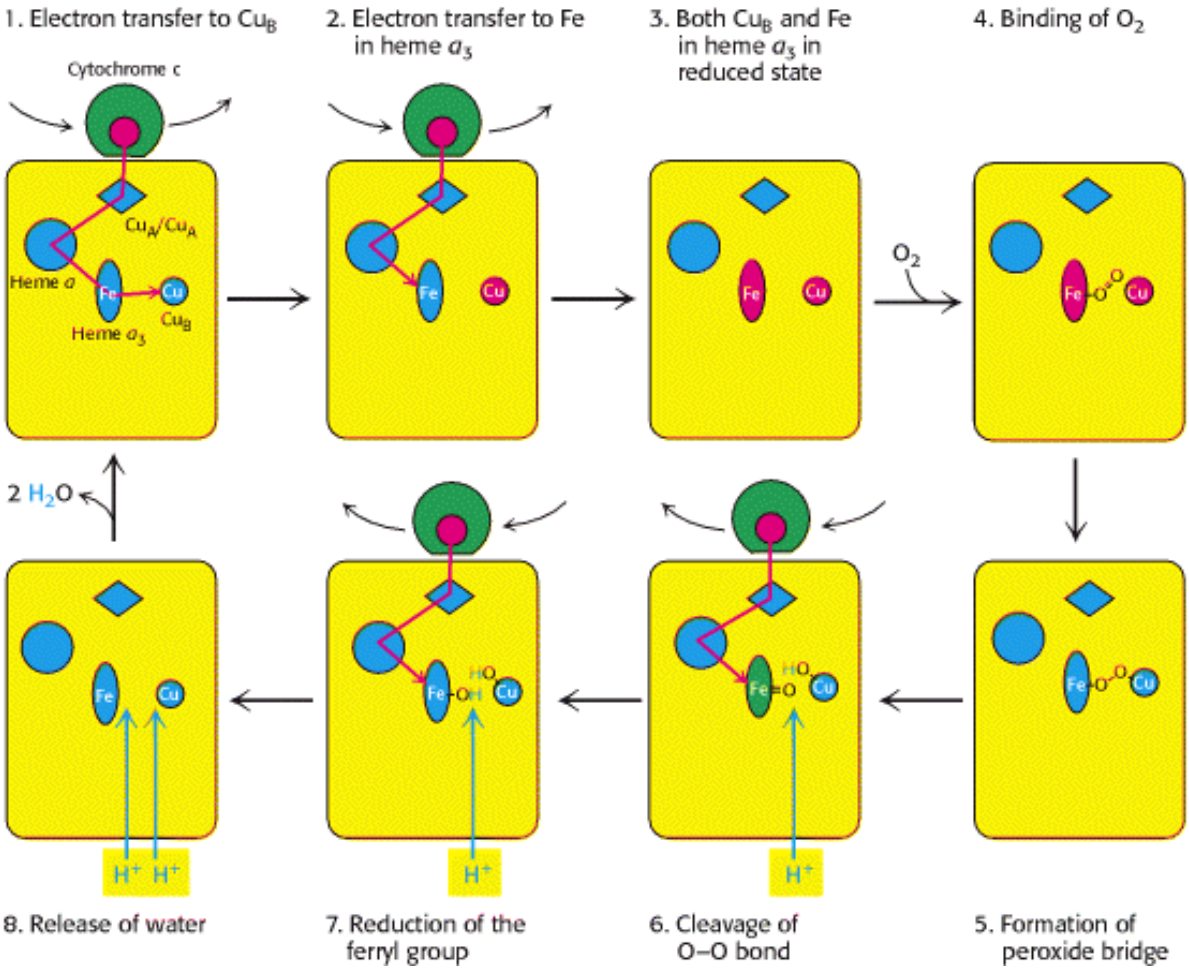


Figure 18.19. Cytochrome Oxidase Mechanism. The cycle begins with all prosthetic groups in their oxidized forms (shown in blue). Reduced cytochrome *c* introduces an electron that reduces Cu_B. A second reduced cytochrome *c* then reduces the iron in heme a_3. This Fe^{2+} center then binds oxygen. Two electrons are transferred to the bound oxygen to form peroxide, which bridges between the iron and Cu_B. The introduction of an additional electron by a third molecule of reduced cytochrome *c* cleaves the O-O bond and results in the uptake of a proton from the matrix. The introduction of a final electron and three more protons generates two molecules of H_2O, which are released from the enzyme to regenerate the initial state. The four protons found in the water molecules come from the matrix.

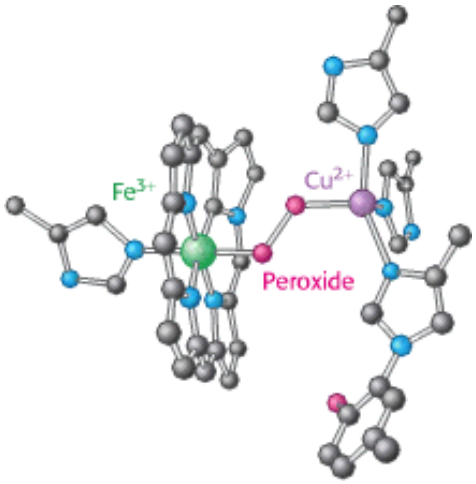


Figure 18.20. Peroxide Bridge. The oxygen bound to heme a_3 is reduced to peroxide by the presence of Cu_B.

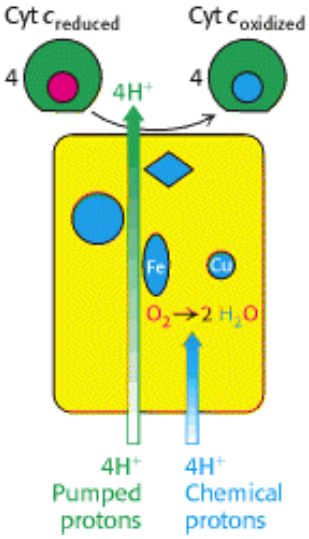


Figure 18.21. Proton Transport by Cytochrome *C* Oxidase. Four "chemical" protons are taken up from the matrix side to reduce one molecule of O_2 to two molecules of H_2O. Four additional "pumped" protons are transported out of the matrix and released on the cytosolic side in the course of the reaction. The pumped protons double the efficiency of free-energy storage in the form of a proton gradient for this final step in the electron-transport chain.

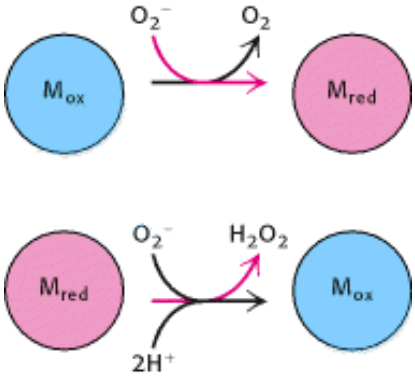


Figure 18.22. Superoxide Dismutase Mechanism. The oxidized form of superoxide dismutase (M_{ox}) reacts with one superoxide ion to form O_2 and generate the reduced form of the enzyme (M_{red}). The reduced form then reacts with a second superoxide and two protons to form hydrogen peroxide and regenerate the oxidized form of the enzyme.

Table 18.3. Pathological and other conditions that may entail free-radical injury

Atherogenesis
Emphysema; bronchitis
Parkinson disease
Duchenne muscular dystrophy
Cervical cancer
Alcoholic liver disease
Diabetes
Acute renal failure

Down syndrome

Retrolental fibroplasia

Cerebrovascular disorders

Ischemia; reperfusion injury

Source: After D.B. Marks, A.D. Marks, and C.M. Smith, *Basic Medical Biochemistry: A Clinical Approach* (Williams & Wilkins, 1996, p. 331).

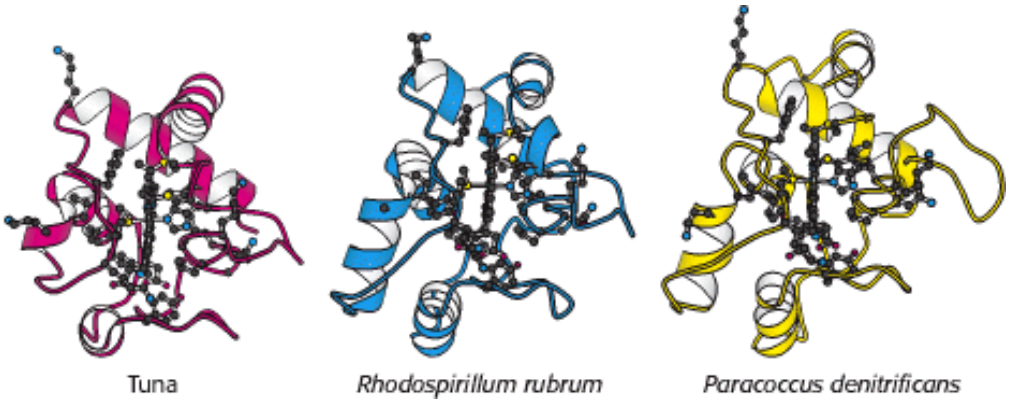


Figure 18.23. Conservation of the Three-Dimensional Structure of Cytochrome *C*. The side chains are shown for the 21 conserved amino acids and the heme.

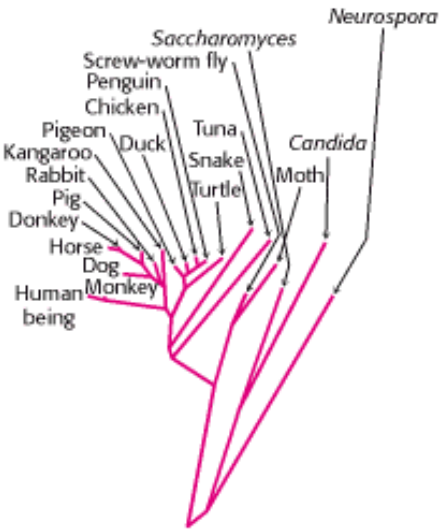


Figure 18.24. Evolutionary Tree Constructed From Sequences of Cytochrome *C*. Branch lengths are proportional to the number of amino acid changes that are believed to have occurred. This drawing is an adaptation of the work of Walter M. Fitch and Emanuel Margoliash.

18.4. A Proton Gradient Powers the Synthesis of ATP

Conceptual Insights, Energy Transformations in Oxidative Phosphorylation. View this media module for an animated, interactive summary of how electron transfer potential is converted into proton-motive force and, finally, phosphoryl transfer potential in oxidative phosphorylation.

Thus far, we have considered the flow of electrons from NADH to O_2, an exergonic process.

$$\text{NADH} + \tfrac{1}{2}\text{O}_2 + \text{H}^+ \rightleftharpoons \text{H}_2\text{O} + \text{NAD}^+$$
$$\Delta G^{\circ\prime} = -52.6 \text{ kcal mol}^{-1} (-220.1 \text{ kJ mol}^{-1})$$

Next, we consider how this process is coupled to the synthesis of ATP, an endergonic process.

$$\text{ADP} + \text{P}_\text{i} + \text{H}^+ \rightleftharpoons \text{ATP} + \text{H}_2\text{O}$$
$$\Delta G^{\circ\prime} = +7.3 \text{ kcal mol}^{-1} (+30.5 \text{ kJ mol}^{-1})$$

A molecular assembly in the inner mitochondrial membrane carries out the synthesis of ATP. This enzyme complex was originally called the *mitochon-drial ATPase* or F_1F_0 *ATPase* because it was discovered through its catalysis of the reverse reaction, the hydrolysis of ATP. *ATP synthase,* its preferred name, emphasizes its actual role in the mitochondrion. It is also called *Complex V.*

How is the oxidation of NADH coupled to the phosphorylation of ADP? It was first suggested that electron transfer leads to the formation of a covalent high-energy intermediate that serves as a high phosphoryl transfer potential compound or to the formation of an activated protein conformation, which then drives ATP synthesis. The search for such intermediates for several decades proved fruitless.

In 1961, Peter Mitchell proposed that electron transport and ATP synthesis are coupled by *a proton gradient across the inner mitochondrial membrane* rather than by a covalent high-energy intermediate or an activated protein conformation. In his model, the transfer of electrons through the respiratory chain leads to the pumping of protons from the matrix to the cytosolic side of the inner mitochondrial membrane. The H^+ concentration becomes lower in the matrix, and an electrical field with the matrix side negative is generated (Figure 18.25). Mitchell's idea, called *the chemiosmotic hypothesis,* was that this proton-motive force drives the synthesis of ATP by ATP synthase. Mitchell's highly innovative hypothesis that oxidation and phosphorylation are coupled by a proton gradient is now supported by a wealth of evidence. Indeed, electron transport does generate a proton gradient across the inner mitochondrial membrane. The pH outside is 1.4 units lower than inside, and the membrane potential is 0.14 V, the outside being positive. As we calculated in Section 18.2.2, this membrane potential corresponds to a free energy of 5.2 kcal (21.8 kJ) per mole of protons.

An artificial system was created to elegantly demonstrate the basic principle of the chemiosmotic hypothesis. Synthetic vesicles containing bacteriorhodopsin, a purple-membrane protein from halobacteria that pumps protons when illuminated, and mitochondrial ATP synthase purified from beef heart were created (Figure 18.26). When the vesicles were exposed to light, ATP was formed. This key experiment clearly showed that *the respiratory chain and ATP synthase are biochemically separate systems, linked only by a proton-motive force.*

18.4.1. ATP Synthase Is Composed of a Proton-Conducting Unit and a Catalytic Unit

Biochemical, electron microscopic, and crystallographic studies of ATP synthase have revealed many details of its structure (Figure 18.27). It is a large, complex membrane-embedded enzyme that looks like a ball on a stick. The 85-Å-

diameter ball, called the F_1 subunit, protrudes into the mitochondrial matrix and contains the catalytic activity of the synthase. In fact, isolated F_1 subunits display ATPase activity. The F_1 subunit consists of five types of polypeptide chains (α_3, β_3, γ, δ, and ε) with the indicated stoichiometry. The α and β subunits, which make up the bulk of the F_1, are arranged alternately in a hexameric ring; they are homologous to one another and are members of the P-loop NTPase family (Section 9.4.1). Both bind nucleotides but only the β subunits participate directly in catalysis. The central stalk consists of two proteins: γ and ε. The γ subunit includes a long α-helical coiled coil that extends into the center of the $\alpha_3\beta_3$ hexamer. *The γ subunit breaks the symmetry of the $\alpha_3\beta_3$ hexamer: each of the β subunits is distinct by virtue of its interaction with a different face of γ.* Distinguishing the three β subunits is crucial for the mechanism of ATP synthesis.

The F_0 subunit is a hydrophobic segment that spans the inner mitochondrial membrane. *F_0 contains the proton channel of the complex.* This channel consists of a ring comprising from 10 to 14 **c** subunits that are embedded in the membrane. A single **a** subunit binds to the outside of this ring. The proton channel depends on both the **a** subunit and the **c** ring. The F_0 and F_1 subunits are connected in two ways, by the central $\gamma\,\varepsilon$ stalk and by an exterior column. The exterior column consists of one **a** subunit, two **b** subunits, and the δ subunit. As will be discussed shortly, we can think of the enzyme as consisting of two functional components: (1) a moving unit, or *rotor,* consisting of the **c** ring and the $\gamma\,\varepsilon$ stalk, and (2) a stationary unit, or *stator,* composed of the remainder of the molecule.

18.4.2. Proton Flow Through ATP Synthase Leads to the Release of Tightly Bound ATP: The Binding-Change Mechanism

Conceptual Insights, ATP Synthase as Motor Protein, looks further into the chemistry and mechanics of ATP synthase rotation.

ATP synthase catalyzes the formation of ATP from ADP and orthophosphate.

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightleftharpoons ATP^{4-} + H_2O$$

The actual substrates are Mg^{2+} complexes of ADP and ATP, as in all known phosphoryl transfer reactions with these nucleotides. A terminal oxygen atom of ADP attacks the phosphorus atom of P_i to form a pentacovalent intermediate, which then dissociates into ATP and H_2O (Figure 18.28). The attacking oxygen atom of ADP and the departing oxygen atom of P_i occupy the apices of a trigonal bipyramid.

How does the flow of protons drive the synthesis of ATP? The results of isotopic-exchange experiments unexpectedly revealed that *enzyme-bound ATP forms readily in the absence of a proton-motive force.* When ADP and P_i were added to ATP synthase in $H_2{}^{18}O$, ^{18}O became incorporated into P_i through the synthesis of ATP and its subsequent hydrolysis (Figure 18.29). The rate of incorporation of ^{18}O into P_i showed that about equal amounts of bound ATP and ADP are in equilibrium at the catalytic site, even in the absence of a proton gradient. However, ATP does not leave the catalytic site unless protons flow through the enzyme. Thus, *the role of the proton gradient is not to form ATP but to release it from the synthase.*

On the basis of these and other observations, Paul Boyer proposed a *binding-change mechanism* for proton-driven ATP synthesis. This proposal states that changes in the properties of the three β subunits allow sequential ADP and P_i binding, ATP synthesis, and ATP release. The concepts of this initial proposal refined by more recent crystallographic and other data yield a satisfying mechanism for ATP synthesis. As already noted, interactions with the γ subunit make the three β subunits inequivalent (Figure 18.30). One β subunit can be in the T, or tight, conformation. This conformation binds ATP with great avidity. Indeed, its affinity for ATP is so high that it will convert bound ADP and P_i into ATP with an equilibrium constant near 1, as indicated by the aforediscussed isotopic-exchange experiments. However, the conformation of this subunit is sufficiently constrained that it cannot release ATP. A second subunit will then be in the

L, or loose, conformation. This conformation binds ADP and P_i. It, too, is sufficiently constrained that it cannot release bound nucleotides. The final subunit will be in the O, or open, form. This form can exist with a bound nucleotide in a structure that is similar to those of the T and L forms, but it can also convert to form a more open conformation and release a bound nucleotide (Figure 18.31). This structure, with one of the three β subunits in an open, nucleotide-free state, as well as one with one of the β subunits in a nucleotide-bound O conformation, have been observed crystallographically.

The interconversion of these three forms can be driven by rotation of the γ subunit (Figure 18.32). Suppose the γ subunit is rotated 120 degrees in a counterclockwise direction (as viewed from the top). This rotation will change the subunit in the T conformation into the O conformation, allowing the subunit to release the ATP that has been formed within it. The subunit in the L conformation will be converted into the T conformation, allowing the transition of bound ADP + P_i into ATP. Finally, the subunit in the O conformation will be converted into the L conformation, trapping the bound ADP and P_i so that they cannot escape. The binding of ADP and P_i to the subunit now in the O conformation completes the cycle. This mechanism suggests that ATP can be synthesized by driving the rotation of the γ subunit in the appropriate direction. Likewise, this mechanism suggests that the hydrolysis of ATP by the enzyme should drive the rotation of the γ subunit in the opposite direction.

18.4.3. The World's Smallest Molecular Motor: Rotational Catalysis

Is it possible to observe the proposed rotation directly? Elegant experiments were performed with the use of a simple experimental system consisting of cloned $\alpha_3 \beta_3 \gamma$ subunits only (Figure 18.33). The β subunits were engineered to contain amino-terminal polyhistidine tags, which have a high affinity for nickel ions. This property of the tags allowed the $\alpha_3 \beta_3$ assembly to be immobilized on a glass surface that had been coated with nickel ions. The γ subunit was linked to a fluorescently labeled actin filament to provide a long segment that could be observed under a fluorescence microscope. Remarkably, the addition of ATP caused the actin filament to rotate unidirectionally in a counterclockwise direction. *The γ subunit was rotating, being driven by the hydrolysis of ATP*. Thus, the catalytic activity of an individual molecule could be observed. The counterclockwise rotation is consistent with the predicted mechanism for hydrolysis because the molecule was viewed from below relative to the view shown in Figure 18.32.

More detailed analysis in the presence of lower concentrations of ATP revealed that the γ subunit rotates in 120-degree increments, with each step corresponding to the hydrolysis of a single ATP molecule. In addition, from the results obtained by varying the length of the actin filament and mea-suring the rate of rotation, the enzyme appears to operate near 100% efficiency; that is, essentially all of the energy released by ATP hydrolysis is converted into rotational motion.

18.4.4. Proton Flow Around the c Ring Powers ATP Synthesis

The direct observation of rotary motion of the γ subunit is strong evidence for the rotational mechanism for ATP synthesis. The last remaining question is: How does proton flow through F_0 drive the rotation of the γ subunit? Howard Berg and George Oster proposed an elegant mechanism that provides a clear answer to this question. The mechanism depends on the structures of the **a** and **c** subunits of F_0 (Figure 18.34). The structure of the **c** subunit was determined both by NMR methods and by x-ray crystallography. Each polypeptide chain forms a pair of α helices that span the membrane. An aspartic acid residue (Asp 61) is found in the middle of the second helix. When Asp 61 is in contact with the hydrophobic part of the membrane, the residue must be in the neutral aspartic acid form, rather than in the charged, aspartate form. From 9 to 12 **c** subunits assemble into a symmetric membrane-spanning ring. Although the structure of the **a** subunit has not yet been experimentally determined, a variety of evidence is consistent with a structure that includes two proton half-channels that do not span the membrane (see Figure 18.34). Thus, protons can pass into either of these channels, but they cannot move completely across the membrane. The **a** subunit directly abuts the ring comprising the **c** subunits, with each half-channel directly interacting with one **c** subunit.

With this structure in mind, we can see how a proton gradient can drive rotation of the **c** ring. Suppose that the Asp 61 residues of the two **c** subunits that are in contact with a half-channel have given up their protons so that they are in the charged aspartate form (Figure 18.35), which is possible because they are in relatively hydrophilic environments inside the half-channel. The **c** ring cannot rotate in either direction, because such a rotation would move a charged aspartate residue into the hydrophobic part of the membrane. A proton can move through either half-channel to protonate one of the aspartate residues. However, it is much more likely to pass through the channel that is connected to the cytosolic side of the membrane because the proton concentration is more than 25 times as high on this side as on the matrix side, owing to the action of the electron-transport-chain proteins. The entry of protons into the cytosolic half-channel is further facilitated by the membrane potential of +0.14 V (positive on the cytoplasmic side), which increases the concentration of protons near the mouth of the cytosolic half-channel. *If the aspartate residue is protonated to its neutral form, the **c** ring can now rotate, but only in a clockwise direction.* Such a rotation moves the newly protonated aspartic acid residue into contact with the membrane, moves the charged aspartate residue from contact with the matrix half-channel to the cytosolic half-channel, and moves a different protonated aspartic acid residue from contact with the membrane to the matrix half-channel. The proton can then dissociate from aspartic acid and move through the half-channel into the proton-poor matrix to restore the initial state. This dissociation is favored by the positive charge on a conserved arginine residue (Arg 210) in the **a** subunit. Thus, *the difference in proton concentration and potential on the two sides of the membrane leads to different probabilities of protonation through the two half-channels, which yields directional rotational motion.* Each proton moves through the membrane by riding around on the rotating **c** ring to exit through the matrix half-channel (Figure 18.36).

The **c** ring is tightly linked to the γ and ε subunits. Thus, as the **c** ring turns, these subunits are turned inside the $\alpha_3\beta_3$ hexamer unit of F_1. The exterior column formed by the two **b** chains and the δ subunit prevent the $\alpha_3\beta_3$ hexamer from rotating. Thus, the proton-gradient-driven rotation of the **c** ring drives the rotation of the γ subunit, which in turn promotes the synthesis of ATP through the binding-change mechanism. Recall that the number of **c** subunits in the **c** ring appears to range between 10 and 14. This number is significant because it determines the number of protons that must be transported to generate a molecule of ATP. Each 360-degree rotation of the γ subunit leads to the synthesis and release of three molecules of ATP. Thus, if there are 10 **c** subunits in the ring (as was observed in a crystal structure of yeast mitochondrial ATP synthase), each ATP generated requires the transport of 10/3 = 3.33 protons. For simplicity, we will assume that 3 protons must flow into the matrix for each ATP formed, but we must keep in mind that the true value may differ.

18.4.5. ATP Synthase and G Proteins Have Several Common Features

The α and β subunits of ATP synthase are members of the P-loop NTPase family of proteins. In Chapter 15, we learned that the signaling properties of other members of this family, the G proteins, depend on their ability to bind nucleoside triphosphates and nucleoside diphosphates with great kinetic tenacity. They do not exchange nucleotides unless they are stimulated to do so by interaction with other proteins. The binding-change mechanism of ATP synthase is a variation on this theme. The three different faces of the γ subunit of ATP synthase interact with the P-loop regions of the β subunits to favor the structures of either the NDP- or NTP-binding forms or to facilitate nucleotide release. The conformational changes take place in an orderly way, driven by the rotation of the γ subunit.

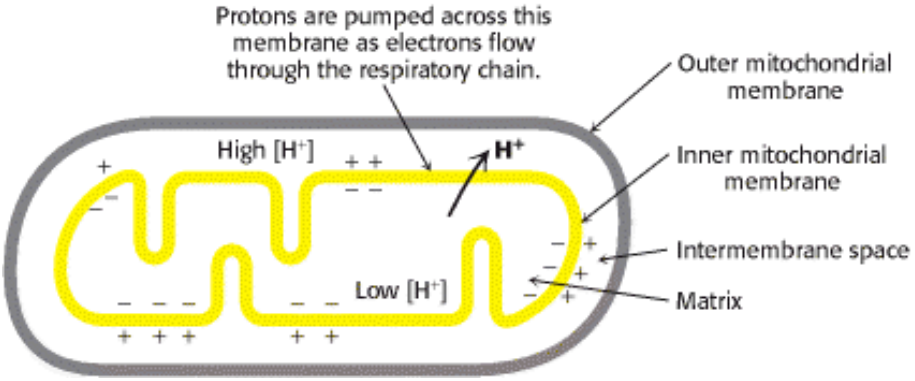


Figure 18.25. Chemiosmotic Hypothesis. Electron transfer through the respiratory chain leads to the pumping of protons from the matrix to the cytosolic side of the inner mitochondrial membrane. The pH gradient and membrane potential constitute a proton-motive force that is used to drive ATP synthesis.

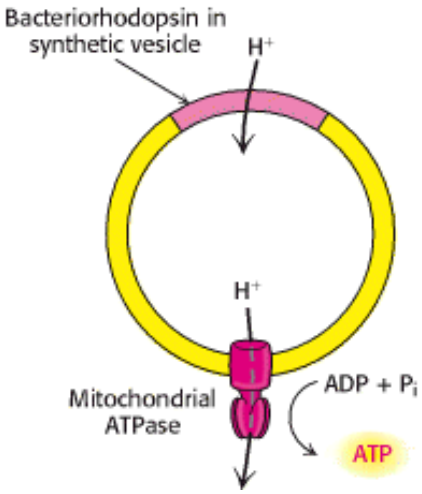


Figure 18.26. Testing the Chemiosmotic Hypothesis. ATP is synthesized when reconstituted membrane vesicles containing bacteriorhodopsin (a light-driven proton pump) and ATP synthase are illuminated. The orientation of ATP synthase in this reconstituted membrane is the reverse of that in the mitochondrion.

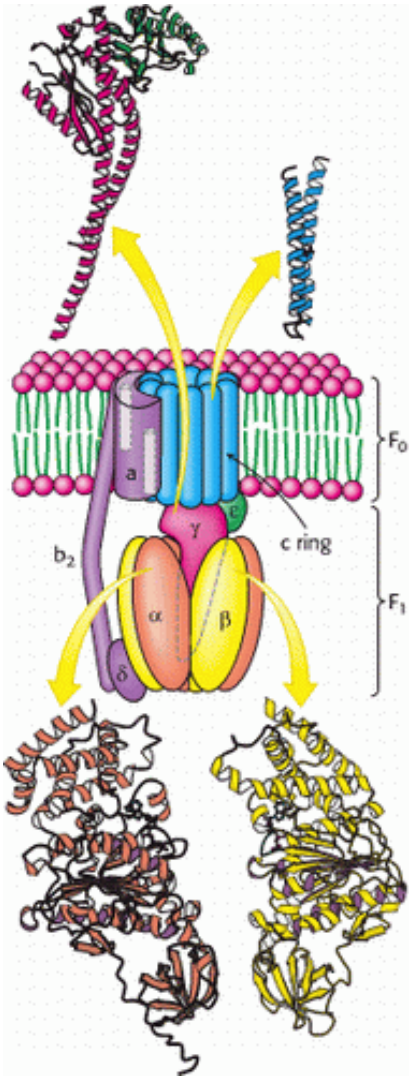


Figure 18.27. Structure of ATP Synthase. A schematic structure is shown along with detailed structures of the components for which structures have been determined to high resolution. The P-loop NTPase domains of the α and β subunits are indicated by purple shading.

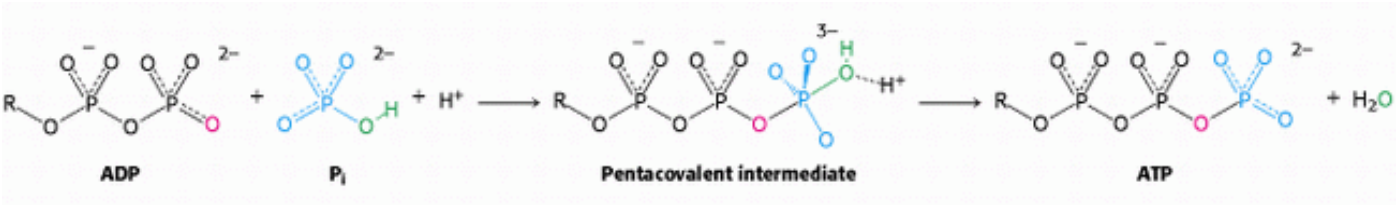


Figure 18.28. ATP Synthesis Mechanism. One of the oxygen atoms of ADP attacks the phosphorus atom of P_i to form a pentacovalent intermediate, which then forms ATP and releases a molecule of H_2O.

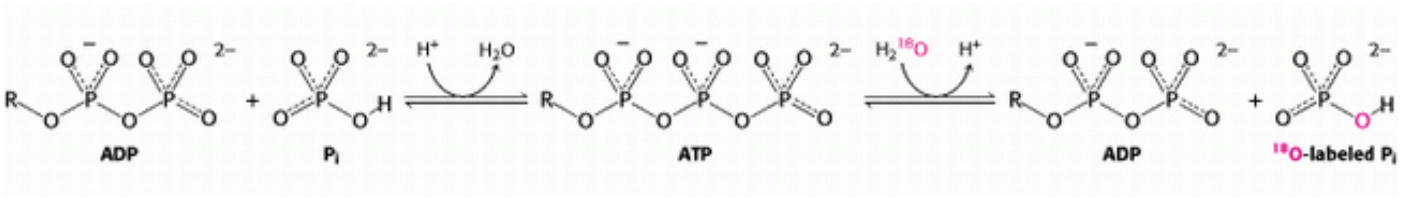


Figure 18.29. ATP Forms Without a Proton-Motive Force But Is Not Released. The results of isotope-exchange experiments indicate that enzyme-bound ATP is formed from ADP and P_i in the absence of a proton-motive force.

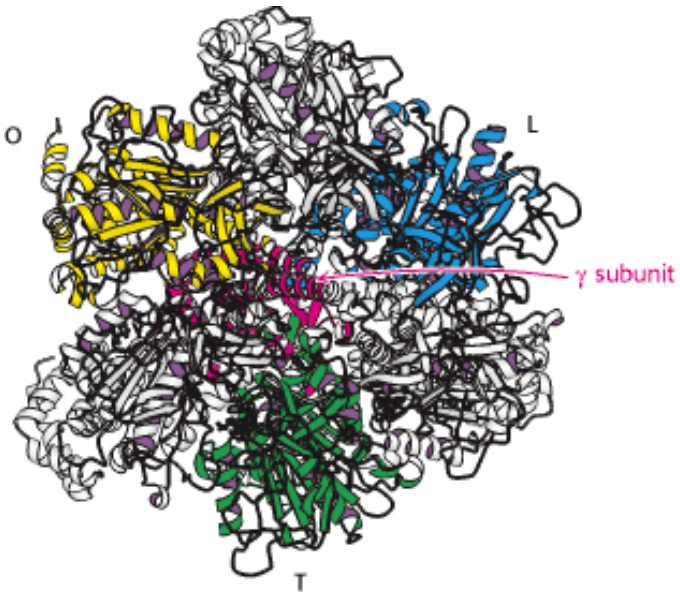


Figure 18.30. ATP Synthase Nucleotide-Binding Sites Are Not Equivalent. The γ subunit passes through the center of the $\alpha_3\beta_3$ hexamer and makes the nucleotide-binding sites in the β subunits distinct from one another.

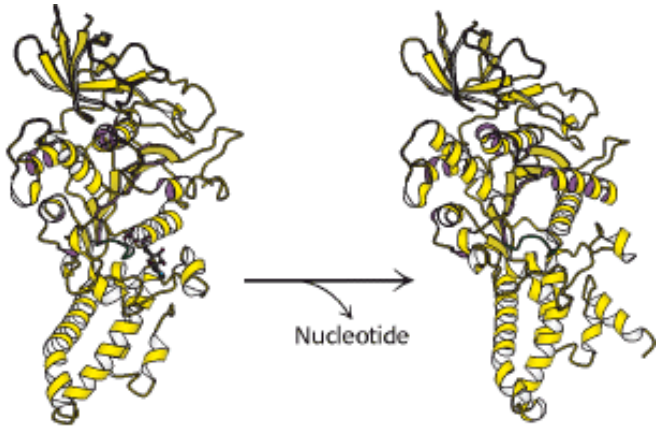


Figure 18.31. ATP Release From the β subunit in the open form. Unlike the tight and loose forms, the open form of the β subunit can change conformation sufficiently to release bound nucleotides.

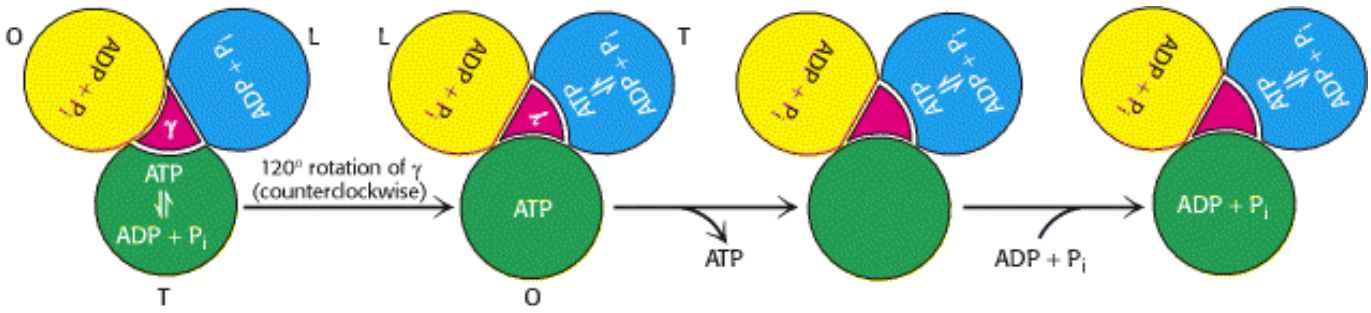


Figure 18.32. Binding-Change Mechanism for ATP Synthase. The rotation of the γ subunit interconverts the three β subunits. The subunit in the T (tight) form, which contains newly synthesized ATP that cannot be released, is converted into the O (open) form. In this form, it can release ATP and then bind ADP and P_i to begin a new cycle.

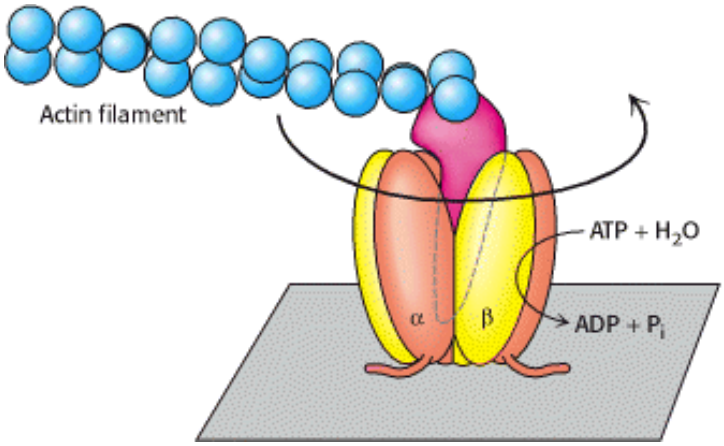


Figure 18.33. Direct Observation of ATP-Driven Rotation in ATP Synthase. The $\alpha_3\beta_3$ hexamer of ATP synthase is fixed to a surface, with the γ subunit projecting upward and linked to a fluorescently labeled actin filament. The addition and subsequent hydrolysis of ATP result in the counterclockwise rotation of the γ subunit, which can be directly seen under a fluorescence microscope.

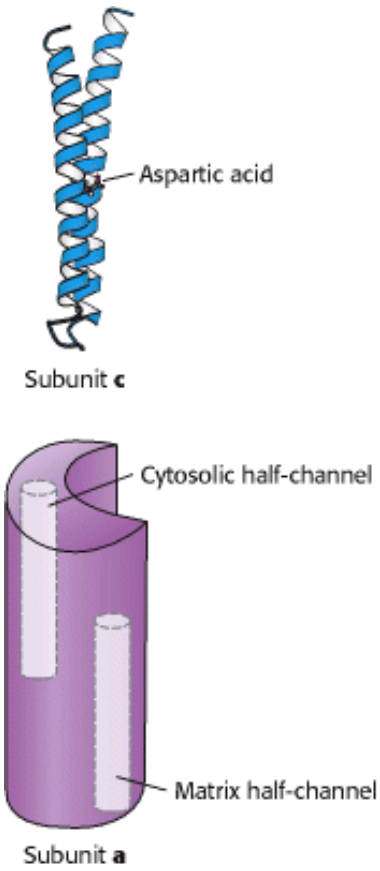


Figure 18.34. Components of the Proton-Conducting Unit of ATP Synthase. The **c** subunit consists of two α helices that span the membrane. An aspartic acid residue in the second helix lies on the center of the membrane. The structure of the **a** subunit has not yet been directly observed, but it appears to include two half-channels that allow protons to enter and pass partway but not completely through the membrane.

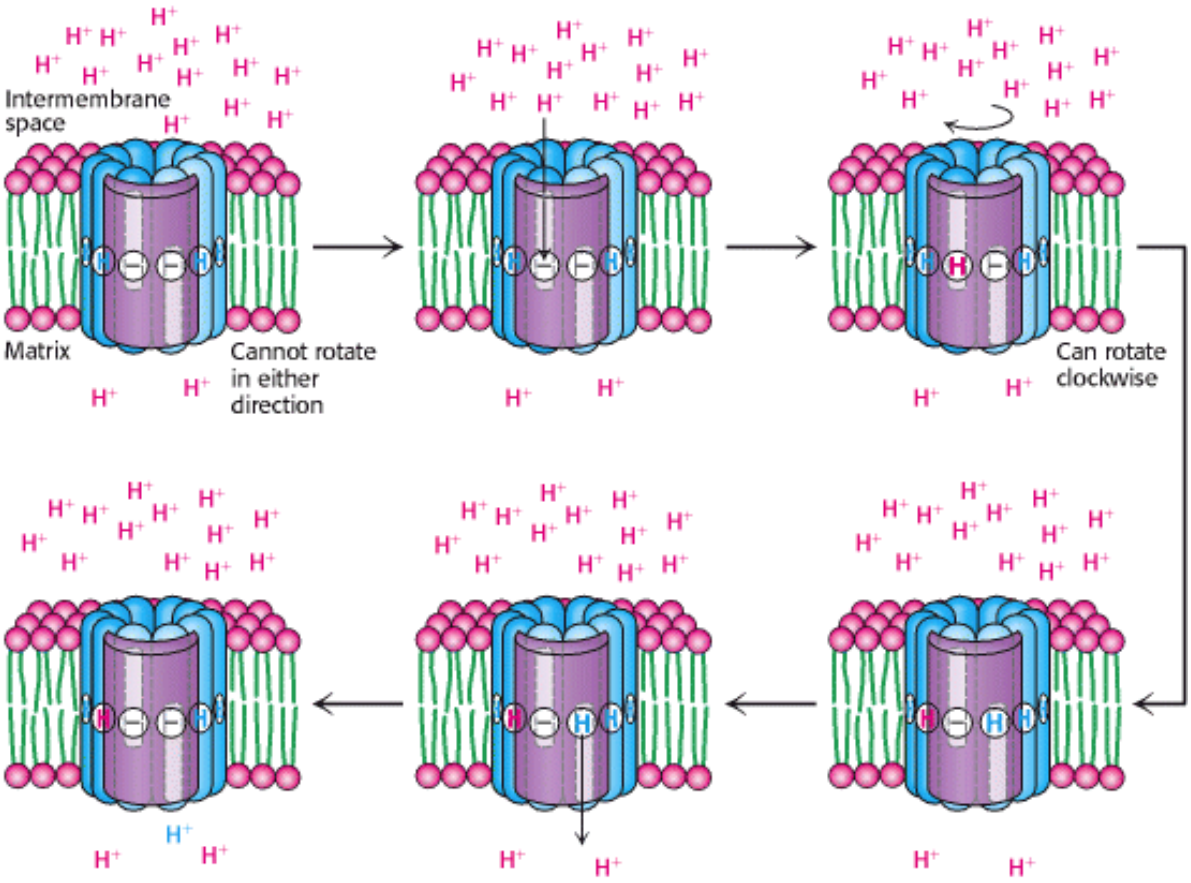


Figure 18.35. Proton Motion Across the Membrane Drives Rotation of the C Ring. A proton enters from the intermembrane space into the cytosolic half-channel to neutralize the charge on an aspartate residue in a **c** subunit. With this charge neutralized, the **c** ring can rotate clockwise by one **c** subunit, moving an aspartic acid residue out of the membrane into the matrix half-channel. This proton can move into the matrix, resetting the system to its initial state.

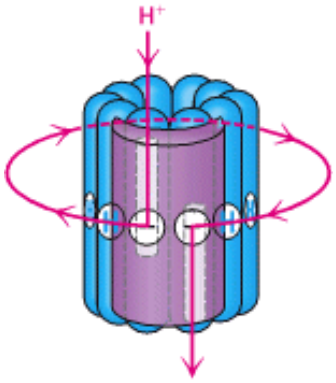


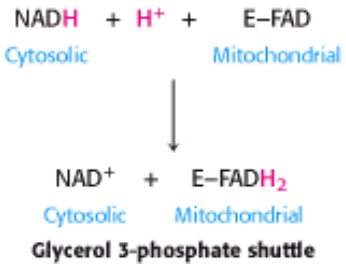
Figure 18.36. Proton Path Through the Membrane. Each proton enters the cytosolic half-channel, follows a complete rotation of the **c** ring, and exits through the other half-channel into the matrix.

18.5. Many Shuttles Allow Movement Across the Mitochondrial Membranes

The inner mitochondrial membrane must be impermeable to most molecules, yet much exchange has to take place between the cytosol and the mitochondria. This exchange is mediated by an array of membrane-spanning transporter proteins (Section 13.4).

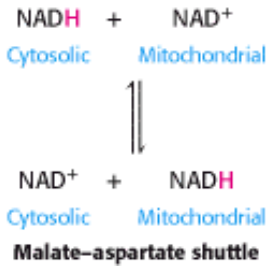
18.5.1. Electrons from Cytosolic NADH Enter Mitochondria by Shuttles

Recall that the glycolytic pathway generates NADH in the cytosol in the oxidation of glyceraldehyde 3-phosphate, and NAD^+ must be regenerated for glycolysis to continue. How is cytosolic NADH reoxidized under aerobic conditions? NADH cannot simply pass into mitochondria for oxidation by the respiratory chain, because the inner mitochondrial membrane is impermeable to NADH and NAD^+. The solution is that *electrons from NADH*, rather than NADH itself, are carried across the mitochondrial membrane. One of several means of introducing electrons from NADH into the electron transport chain is the *glycerol 3-phosphate shuttle* (Figure 18.37). The first step in this shuttle is the transfer of a pair of electrons from NADH to dihydroxyacetone phosphate, a glycolytic intermediate, to form glycerol 3-phosphate.This reaction is catalyzed by a glycerol 3-phosphate dehydrogenase in the cytosol. Glycerol 3-phosphate is reoxidized to dihydroxyacetone phosphate on the outer surface of the inner mitochondrial membrane by a membrane-bound isozyme of glycerol 3-phosphate dehydrogenase. An electron pair from glycerol 3-phosphate is transferred to a FAD prosthetic group in this enzyme to form $FADH_2$. This reaction also regenerates dihydroxyacetone phosphate.



The reduced flavin transfers its electrons to the electron carrier Q, which then enters the respiratory chain as QH_2. *When cytosolic NADH transported by the glycerol 3-phosphate shuttle is oxidized by the respiratory chain, 1.5 rather than 2.5 ATP are formed.* The yield is lower because FAD rather than NAD^+ is the electron acceptor in mitochondrial glycerol 3-phosphate dehydrogenase. The use of FAD enables electrons from cytosolic NADH to be transported into mitochondria against an NADH concentration gradient. The price of this transport is one molecule of ATP per two electrons. This glycerol 3-phosphate shuttle is especially prominent in muscle and enables it to sustain a very high rate of oxidative phosphorylation. Indeed, some insects lack lactate dehydrogenase and are completely dependent on the glycerol 3-phosphate shuttle for the regeneration of cytosolic NAD^+.

In the heart and liver, electrons from cytosolic NADH are brought into mitochondria by the *malate-aspartate shuttle*, which is mediated by two membrane carriers and four enzymes (Figure 18.38). Electrons are transferred from NADH in the cytosol to oxaloacetate, forming malate, which traverses the inner mitochondrial membrane and is then reoxidized by NAD^+ in the matrix to form NADH in a reaction catalyzed by the citric acid cycle enzyme malate dehydrogenase. The resulting oxaloacetate does not readily cross the inner mitochondrial membrane, and so a transamination reaction (Section 23.3.1) is needed to form aspartate, which can be transported to the cytosolic side. Mitochondrial glutamate donates an amino group, forming aspartate and α -ketoglutarate. In the cytoplasm, aspartate is then deaminated to form oxaloacetate and the cycle is restarted. This shuttle, in contrast with the glycerol 3-phosphate shuttle, is readily reversible. Consequently, NADH can be brought into mitochondria by the malate- aspartate shuttle only if the NADH/ NAD^+ ratio is higher in the cytosol than in the mitochondrial matrix. This versatile shuttle also facilitates the exchange of key intermediates between mitochondria and the cytosol.



Malate–aspartate shuttle

18.5.2. The Entry of ADP into Mitochondria Is Coupled to the Exit of ATP by ATP-ADP Translocase

The major function of oxidative phosphorylation is to generate ATP from ADP. However, ATP and ADP do not diffuse freely across the inner mitochondrial membrane. How are these highly charged molecules moved across the inner membrane into the cytosol? A specific transport protein, *ATP-ADP translocase* (also called *adenine nucleotide translocase* or *ANT*), enables these molecules to traverse this permeability barrier. Most important, the flows of ATP and ADP are coupled. *ADP enters the mitochondrial matrix only if ATP exits, and vice versa.* The reaction catalyzed by the translocase, which acts as an antiporter, is

$$ADP^{3-}_{cytosol} + ATP^{4-}_{matrix} \longrightarrow ADP^{3-}_{matrix} + ATP^{4-}_{cytosol}$$

ATP-ADP translocase is highly abundant, constituting about 14% of the protein in the inner mitochondrial membrane. The translocase, a dimer of identical 30-kd subunits, contains a single nucleotide-binding site that alternately faces the matrix and cytosolic sides of the membrane (Figure 18.39). ATP and ADP (both devoid of Mg^{2+}) are bound with nearly the same affinity. In the presence of a positive membrane potential (as would be the case for an actively respiring mitochondrion), *the rate of binding-site eversion from the matrix to the cytosolic side is more rapid for ATP than for ADP because ATP has one more negative charge.* Hence, ATP is transported out of the matrix about 30 times as rapidly as is ADP, which leads to a higher phosphoryl transfer potential on the cytosolic side than on the matrix side. The translocase does not evert at an appreciable rate unless a molecule of ADP is bound at the open, cytosolic site, which then everts to the mitochondrial matrix side. This feature ensures that the entry of ADP into the matrix is precisely coupled to the exit of ATP. The other side of the coin is that *the membrane potential and hence the proton-motive force are decreased by the exchange of ATP for ADP, which results in a net transfer of one negative charge out of the matrix.* ATP-ADP exchange is energetically expensive; about a quarter of the energy yield from electron transfer by the respiratory chain is consumed to regenerate the membrane potential that is tapped by this exchange process. The inhibition of this process leads to the subsequent inhibition of cellular respiration as well (Section 18.6.3).

18.5.3. Mitochondrial Transporters for Metabolites Have a Common Tripartite Motif

ATP-ADP translocase is but one of many mitochondrial transporters for ions and charged metabolites (Figure 18.40). For historical reasons, these transmembrane proteins are sometimes called carriers. Recall that some of them function as symporters and others as antiporters (Section 13.4). The *phosphate carrier*, which works in concert with ATP-ADP translocase, mediates the electroneutral exchange of $H_2PO_4^-$ for OH^- (or, indistinguishably, the electroneutral symport of $H_2PO_4^-$ and H^+). The combined action of these two transporters leads to the exchange of cytosolic ADP and P_i for matrix ATP at the cost of an influx of one H^+. The *dicarboxylate carrier* enables malate, succinate, and fumarate to be exported from mitochondria in exchange for P_i. The *tricarboxylate carrier* transports citrate and H^+ in exchange for malate. Pyruvate in the cytosol enters the mitochondrial matrix in exchange for OH^- (or together with H^+) by means of the *pyruvate carrier.* These mitochondrial transporters and more than five others have a common structural motif. They are constructed from three tandem repeats of a 100-residue module, each containing two putative transmembrane segments (Figure 18.41).

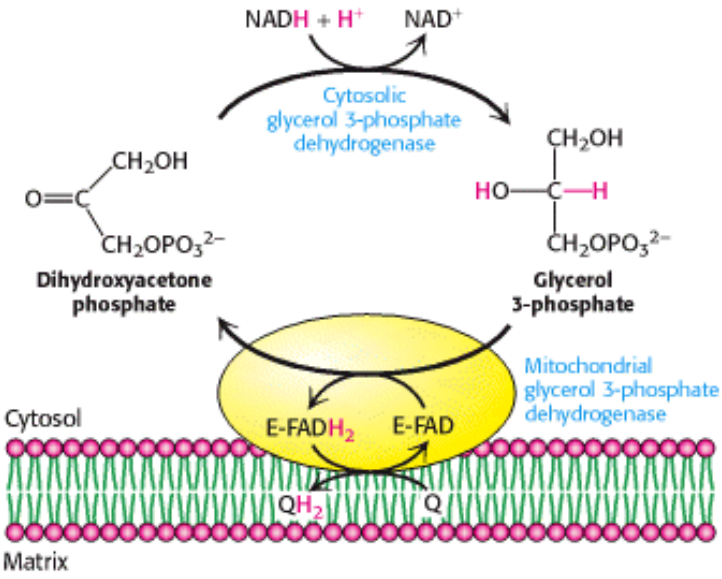


Figure 18.37. Glycerol 3-Phosphate Shuttle. Electrons from NADH can enter the mitochondrial electron transport chain by being used to reduce dihydroxyacetone phosphate to glycerol 3-phosphate. Glycerol 3-phosphate is reoxidized by electron transfer to an FAD prosthetic group in a membrane-bound glycerol 3-phosphate dehydrogenase. Subsequent electron transfer to Q to form QH_2 allows these electrons to enter the electron-transport chain.

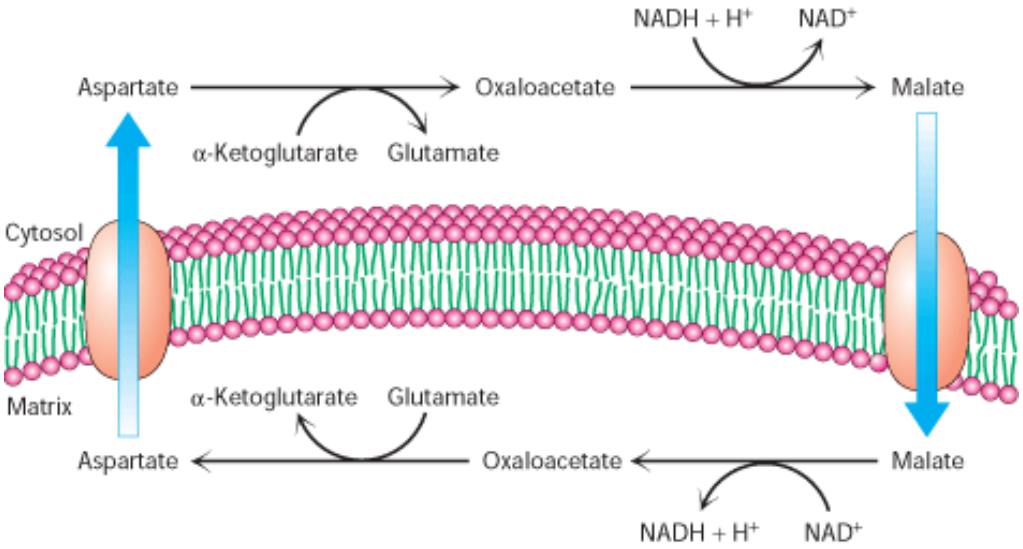


Figure 18.38. Malate-Aspartate Shuttle.

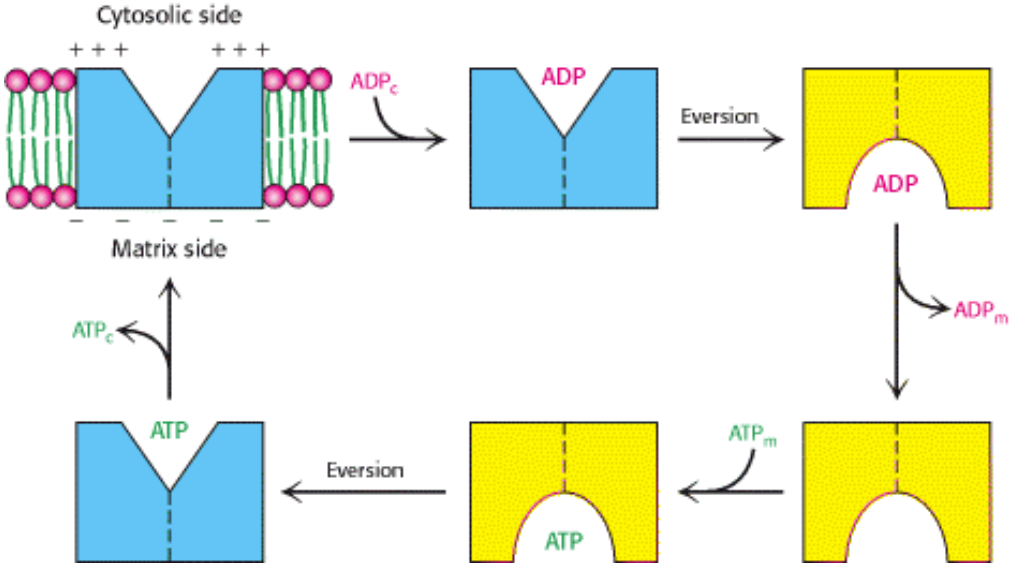


Figure 18.39. Mechanism of Mitochondrial ATP-ADP Translocase. The translocase catalyzes the coupled entry of ADP and exit of ATP into and from the matrix. The reaction cycle is driven by membrane potential. The actual conformational change corresponding to eversion of the binding site could be quite small.

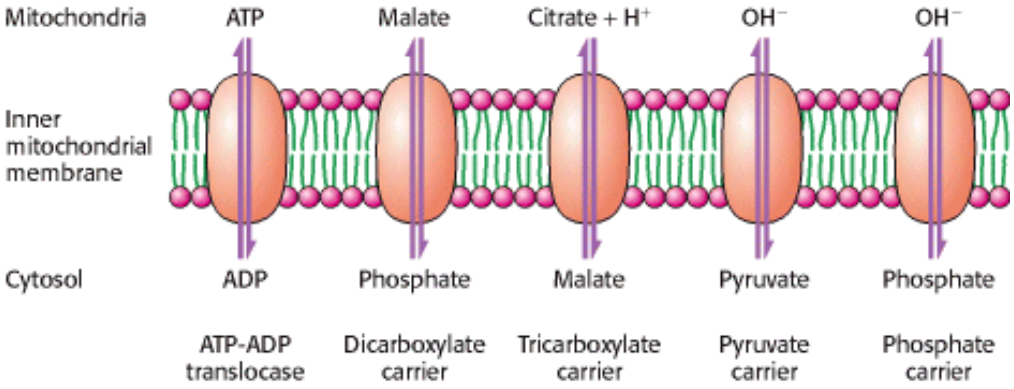


Figure 18.40. Mitochondrial Transporters. Transporters (also called carriers) are transmembrane proteins that move ions and charged metabolites across the inner mitochondrial membrane.

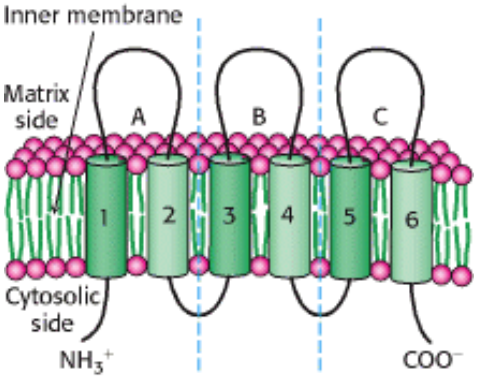


Figure 18.41. Structure of Mitochondrial Transporters. Many mitochondrial transporters consist of three similar 100-residue units. These proteins contain six putative membrane-spanning segments. [After J. E. Walker. *Curr. Opin. Struct. Biol*. 2(1992):519.]

18.6. The Regulation of Cellular Respiration Is Governed Primarily by the Need for ATP

Because ATP is the end product of cellular respiration, its concentration is the ultimate determinant of the rate of all of the components of respiratory pathways.

18.6.1. The Complete Oxidation of Glucose Yields About 30 Molecules of ATP

We can now estimate how many molecules of ATP are formed when glucose is completely oxidized to CO_2. The number of ATP (or GTP) molecules formed in glycolysis and the citric acid cycle is unequivocally known because it is determined by the stoichiometries of chemical reactions. In contrast, the ATP yield of oxidative phosphorylation is less certain because the stoichiometries of proton pumping, ATP synthesis, and metabolite transport processes need not be integer numbers or even have fixed values. As discussed earlier, the best current estimates for the number of protons pumped out of the matrix by NADH-Q oxidoreductase, Q-cytochrome *c* oxidoreductase, and cytochrome *c* oxidase per electron pair are four, two, and four, respectively. The synthesis of a molecule of ATP is driven by the flow of about three protons through ATP synthase. An additional proton is consumed in transporting ATP from the matrix to the cytosol. Hence, about 2.5 molecules of cytosolic ATP are generated as a result of the flow of a pair of electrons from NADH to O_2. For electrons that enter at the level of Q-cytochrome *c* oxidoreductase, such as those from the oxidation of succinate or cytosolic NADH, the yield is about 1.5 molecules of ATP per electron pair. Hence, as tallied in Table 18.4, *about 30 molecules of ATP are formed when glucose is completely oxidized to* CO_2*;* this value supersedes the traditional estimate of 36 molecules of ATP. Most of the ATP, 26 of 30 molecules formed, is generated by oxidative phosphorylation. Recall that the anaerobic metabolism of glucose yields only 2 molecules of ATP.

18.6.2. The Rate of Oxidative Phosphorylation Is Determined by the Need for ATP

How is the rate of the electron-transport chain controlled? Under most physiological conditions, electron transport is tightly coupled to phosphory-lation. *Electrons do not usually flow through the electron-transport chain to* O_2 *unless ADP is simultaneously phosphorylated to ATP*. Oxidative phosphorylation requires a supply of NADH (or other source of electrons at high potential), O_2, ADP, and P_i. The most important factor in determining the rate of oxidative phosphorylation is the *level of ADP*. The rate of oxygen consumption by mitochondria increases markedly when ADP is added and then returns to its initial value when the added ADP has been converted into ATP (Figure 18.42).

The regulation of the rate of oxidative phosphorylation by the ADP level is called *respiratory control* or *acceptor control.* The level of ADP likewise affects the rate of the citric acid cycle because of its need for NAD^+ and FAD. The physiological significance of this regulatory mechanism is evident. The ADP level increases when ATP is consumed, and so oxidative phosphorylation is coupled to the utilization of ATP. *Electrons do not flow from fuel molecules to* O_2 *unless ATP needs to be synthesized.* We see here another example of the regulatory significance of the energy charge.

18.6.3. Oxidative Phosphorylation Can Be Inhibited at Many Stages

Oxidative phosphorylation is susceptible to inhibition at all stages of the process. Specific inhibitors of electron transport were invaluable in revealing the sequence of electron carriers in the respiratory chain. For example, *rotenone* and *amytal* block electron transfer in NADH-Q oxidoreductase and thereby prevent the utilization of NADH as a substrate (Figure 18.43). In contrast, electron flow resulting from the oxidation of succinate is unimpaired, because these electrons enter through QH_2, beyond the block. *Antimycin A* interferes with electron flow from cytochrome b_H in Q-cytochrome *c* oxidoreductase. Furthermore, electron flow in cytochrome *c* oxidase can be blocked by *cyanide* (CN^-), *azide* (N_3^-), and *carbon monoxide* (CO). Cyanide and azide react with the ferric form of heme a_3, whereas carbon monoxide inhibits the

ferrous form. Inhibition of the electron-transport chain also inhibits ATP synthesis because the proton-motive force can no longer be generated.

ATP synthase also can be inhibited. Oligomycin and dicyclohexylcarbodiimide (DCCD) prevent the influx of protons through ATP synthase. If actively respiring mitochondria are exposed to an inhibitor of ATP synthase, the electron-transport chain ceases to operate. Indeed, this observation clearly illustrates that electron transport and ATP synthesis are normally tightly coupled.

This tight coupling of electron transport and phosphorylation in mitochondria can be disrupted (uncoupled) by 2,4-dinitrophenol (Figure 18.44) *and certain other acidic aromatic compounds. These substances carry protons across the inner mitochondrial membrane.* In the presence of these uncouplers, electron transport from NADH to O_2 proceeds in a normal fashion, but ATP is not formed by mitochondrial ATP synthase because the proton-motive force across the inner mitochondrial membrane is dissipated. This loss of respiratory control leads to increased oxygen consumption and oxidation of NADH. Indeed, in the accidental ingestion of uncouplers, large amounts of metabolic fuels are consumed, but no energy is stored as ATP. Rather, energy is released as heat. DNP and other uncouplers are very useful in metabolic studies because of their specific effect on oxidative phosphorylation. The regulated uncoupling of oxidative phosphorylation is a biologically useful means of generating heat.

ATP-ADP translocase is specifically inhibited by very low concentrations of *atractyloside* (a plant glycoside) or *bongkrekic acid* (an antibiotic from a mold). Atractyloside binds to the translocase when its nucleotide site faces the cytosol, whereas bongkrekic acid binds when this site faces the mitochondrial matrix. Oxidative phosphorylation stops soon after either inhibitor is added, showing that ATP-ADP translocase is essential.

18.6.4. Regulated Uncoupling Leads to the Generation of Heat

The uncoupling of oxidative phosphorylation is a means of generating heat to maintain body temperature in hibernating animals, in some newborn animals (including human beings), and in mammals adapted to cold. Brown adipose tissue, which is very rich in mitochondria (often referred to as brown fat mitochondria), is specialized for this process of *nonshivering thermogenesis.* The inner mitochondrial membrane of these mitochondria contains a large amount of *uncoupling protein (UCP)*, here UCP-1, or *thermogenin*, a dimer of 33-kd subunits that resembles ATP-ADP translocase. UCP-1 forms a pathway for the flow of protons from the cytosol to the matrix. In essence, *UCP-1 generates heat by short-circuiting the mitochondrial proton battery.* This dissipative proton pathway is activated by free fatty acids liberated from triacylglycerols in response to hormonal signals, such as β-adrenergic agonists (Figure 18.45).

In addition to UCP-1, two other uncoupling proteins have been identified. UCP-2, which is 56% identical in sequence with UCP-1, is found in a wide variety of tissues. UCP-3 (57% identical with UCP-1 and 73% identical with UCP-2) is localized to skeletal muscle and brown fat. This family of uncoupling proteins, especially UCP-2 and UCP-3, may play a role in energy homeostasis. In fact, the genes for UCP-2 and UCP-3 map to regions of the human and mouse chromosomes that have been linked to obesity, substantiating the notion that they function as a means of regulating body weight. The use of uncoupling proteins is not limited to animals, however. The skunk cabbage uses an analogous mechanism to heat its floral spikes, increasing the evaporation of odoriferous molecules that attract insects to fertilize its flowers.

18.6.5. Mitochondrial Diseases Are Being Discovered

As befitting an organelle that is so central to energy metabolism, mitochondrial malfunction can lead to pathological conditions. The number of diseases that can be attributed to mitochondrial mutations is steadily growing in step with our growing understanding of the biochemistry and genetics of mitochondria. The first mitochondrial disease to be understood was Leber hereditary optic neuropathy (LHON), a form of blindness that strikes in midlife as a result of mutations to the NADH-Q oxidoreductase component of Complex I. Some of these mutations impair NADH utilization, whereas others block electron transfer to Q. The accumulation of mutations in mitochondrial genes in the course of several decades may contribute to aging, degenerative disorders, and cancer.

A human egg harbors several hundred thousand molecules of mitochondrial DNA, whereas a sperm contributes only a few hundred and thus has little effect on the mitochondrial genotype. Because the maternally inherited mitochondria are present in large numbers and not all of the mitochondria may be affected, the pathologies of mitochondrial mutants can be quite complex. Even within a single family carrying an identical mutation, chance fluctuations in the percentage of mitochondria with the mutation lead to large variations in the nature and severity of the symptoms of the pathological condition as well as the time of onset. As the percentage of defective mitochondria increases, energy-generating capacity diminishes until, at some threshold, the cell can no longer function properly. Defects in cellular respiration are doubly dangerous. Not only does energy transduction decrease, but also the likelihood that reactive oxygen species will be generated increases. Organs that are highly dependent on oxidative phosphorylation, such as the nervous system and the heart, are most vulnerable to mutations in mitochondrial DNA.

18.6.6. Mitochondria Play a Key Role in Apoptosis

In the course of development or in cases of significant cell damage, individual cells within multicellular organisms undergo *programmed cell death*, or *apoptosis*. Mitochondria act as control centers regulating this process. Although the details have not yet been established, a pore called the mitochondrial permeability transition pore (mtPTP) forms in damaged mitochondria. This pore appears to consist of VDAC (the adenine nucleotide translocator) and several other mitochondrial proteins, including members of a family of proteins (Bcl family) that were initially discovered because of their role in cancer. One of the most potent activators of apoptosis is cytochrome *c*. Its presence in the cytosol activates a cascade of proteolytic enzymes called *caspases*. These cysteine proteases (Section 9.1.6) are conserved in evolution, being found in organisms ranging from hydra to human beings. Cytochrome *c*, in conjunction with other proteins, initiates the cascade by activating procaspase 9 to form caspase 9, which then activates other caspases. Activation of the caspase cascade does not lead to generalized protein destruction. Rather, the caspases have particular targets. For instance, the proteins that maintain cell structure are destroyed. Another example is the degradation of a protein that inhibits an enzyme that destroys DNA (caspase-activated DNAse, CAD), freeing CAD to cleave the genetic material. This cascade of proteolytic enzymes has been called "death by a thousand tiny cuts."

18.6.7. Power Transmission by Proton Gradients: A Central Motif of Bioenergetics

The main concept presented in this chapter is that mitochondrial electron transfer and ATP synthesis are linked by a transmembrane proton gradient. ATP synthesis in bacteria and chloroplasts (Section 19.4) also is driven by proton gradients. In fact, proton gradients power a variety of energy-requiring processes such as the active transport of calcium ions by mitochondria, the entry of some amino acids and sugars into bacteria, the rotation of bacterial flagella, and the transfer of electrons from $NADP^+$ to NADPH. Proton gradients can also be used to generate heat, as in hibernation. It is evident that *proton gradients are a central interconvertible currency of free energy in biological systems* (Figure 18.46). Mitchell noted that the proton-motive force is a marvelously simple and effective store of free energy because it requires only a thin, closed lipid membrane between two aqueous phases.

Table 18.4. ATP yield from the complete oxidation of glucose

Reaction sequence	ATP yield per glucose molecule
Glycolysis: Conversion of glucose into pyruvate (in the cytosol)	
Phosphorylation of glucose	- 1
Phosphorylation of fructose 6-phosphate	- 1
Dephosphorylation of 2 molecules of 1,3-BPG	+ 2
Dephosphorylation of 2 molecules of phosphoenolpyruvate	+ 2

2 molecules of NADH are formed in the oxidation of 2 molecules of glyceraldehyde 3-phosphate	
Conversion of pyruvate into acetyl CoA (inside mitochondria)	
2 molecules of NADH are formed	
Citric acid cycle (inside mitochondria)	
2 molecules of guanosine triphosphate are formed from 2 molecules of succinyl CoA	+ 2
6 molecules of NADH are formed in the oxidation of 2 molecules each of isocitrate, α-ketoglutarate, and malate	
2 molecules of $FADH_2$ are formed in the oxidation of 2 molecules of succinate	
Oxidative phosphorylation (inside mitochondria)	
2 molecules of NADH formed in glycolysis; each yields 1.5 molecules of ATP (assuming transport of NADH by the glycerol 3-phosphate shuttle)	+ 3
2 molecules of NADH formed in the oxidative decarboxylation of pyruvate; each yields 2.5 molecules of ATP	+ 5
2 molecules of $FADH_2$ formed in the citric acid cycle; each yields 1.5 molecules of ATP	+ 3
6 molecules of NADH formed in the citric acid cycle; each yields 2.5 molecules of ATP	+ 15
NET YIELD PER MOLECULE OF GLUCOSE	+ 30

Source: The ATP yield of oxidative phosphorylation is based on values given in P. C. Hinkle, M. A. Kumar, A. Resetar, and D. L. Harris, *Biochemistry* 30(1991):3576.

Note: The current value of 30 molecules of ATP per molecule of glucose supersedes the earlier one of 36 molecules of ATP. The stoichiometries of proton pumping, ATP synthesis, and metabolite transport should be regarded as estimates. About two more molecules of ATP are formed per molecule of glucose oxidized when the malate-aspartate shuttle rather than the glycerol 3-phosphate shuttle is used.

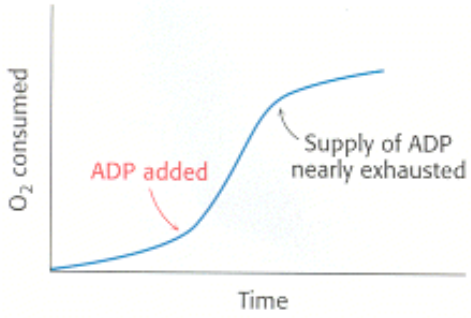


Figure 18.42. Respiratory Control. Electrons are transferred to O_2 only if ADP is concomitantly phosphorylated to ATP.

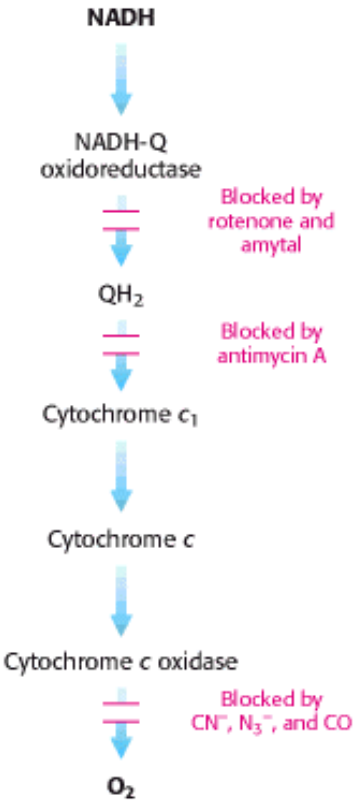


Figure 18.43. Sites of Action of Some Inhibitors of Electron Transport.

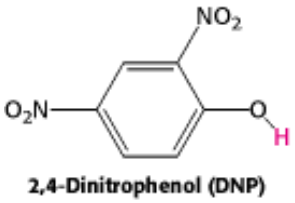


Figure 18.44. Uncoupler of Oxidative Phosphorylation. 2,4-Dinitrophenol, a lipid-soluble substance, can carry protons across the inner mitochondrial membrane. The dissociable proton is shown in red.

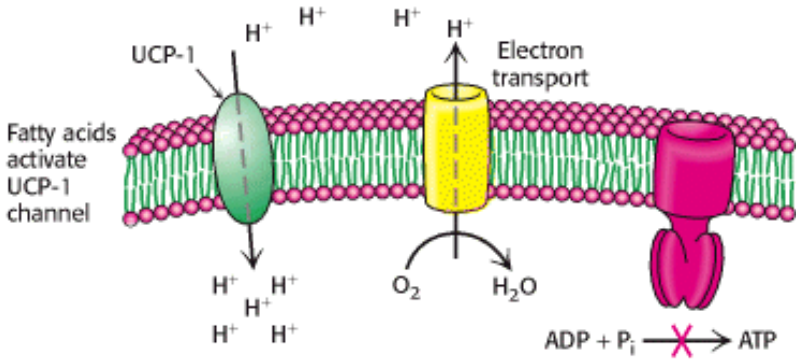


Figure 18.45. Action of an Uncoupling Protein. Uncoupling protein-1 (UCP-1) generates heat by permitting the influx of protons into the mitochondria without the synthesis of ATP.

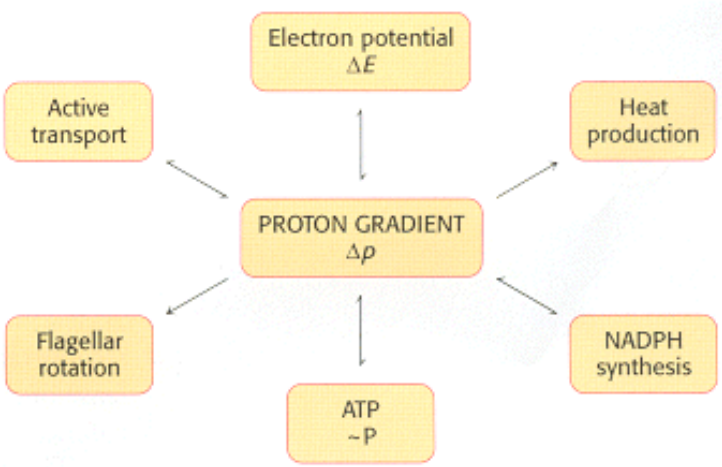


Figure 18.46. The Proton Gradient Is an Interconvertible Form of Free Energy.

Summary

Oxidative Phosphorylation in Eukaryotes Takes Place in Mitochondria

Mitochondria generate most of the ATP required by aerobic cells by a joint endeavor of the reactions of citric acid cycle, which take place in the mitochondrial matrix, and oxidative phosphorylation, which takes place in the inner mitochondrial membrane. Mitochondria are descendents of a free-living bacterium that established a symbiotic relation with another cell.

Oxidative Phosphorylation Depends on Electron Transfer

In oxidative phosphorylation, the synthesis of ATP is coupled to the flow of electrons from NADH or $FADH_2$ to O_2 by a proton gradient across the inner mitochondrial membrane. Electron flow through three asymmetrically oriented transmembrane complexes results in the pumping of protons out of the mitochondrial matrix and the generation of a membrane potential. ATP is synthesized when protons flow back to the matrix through a channel in an ATP-synthesizing complex, called ATP synthase (also known as F_0F_1-ATPase). Oxidative phosphorylation exemplifies a fundamental theme of bioenergetics: the transmission of free energy by proton gradients.

The Respiratory Chain Consists of Four Complexes: Three Proton Pumps and a Physical Link to the Citric Acid Cycle

The electron carriers in the respiratory assembly of the inner mitochondrial membrane are quinones, flavins, iron-sulfur complexes, heme groups of cytochromes, and copper ions. Electrons from NADH are transferred to the FMN prosthetic group of NADH-Q oxidoreductase (Complex I), the first of four complexes. This oxidoreductase also contains Fe-S centers. The electrons emerge in QH_2, the reduced form of ubiquinone (Q). The citric acid cycle enzyme succinate dehydrogenase is a component of the succinate-Q reductase complex (Complex II), which donates electrons from $FADH_2$ to Q to form QH_2.This highly mobile hydrophobic carrier transfers its electrons to Q-cytochrome *c* oxidoreductase (Complex III), a complex that contains cytochromes *b* and c_1 and an Fe-S center. This complex reduces cytochrome *c*, a water-soluble peripheral membrane protein. Cytochrome *c*, like Q, is a mobile carrier of electrons, which it then transfers to cytochrome *c* oxidase (Complex IV). This complex contains cytochromes *a* and a_3 and three copper ions. A heme iron ion and a copper ion in this oxidase transfer electrons to O_2, the ultimate acceptor, to form H_2O.

A Proton Gradient Powers the Synthesis of ATP

The flow of electrons through Complexes I, III, and IV leads to the transfer of protons from the matrix side to the cytosolic side of the inner mitochondrial membrane. A proton-motive force consisting of a pH gradient (matrix side basic) and a membrane potential (matrix side negative) is generated. The flow of protons back to the matrix side through ATP synthase drives ATP synthesis. The enzyme complex is a molecular motor made of two operational units: a rotating component and a stationary component. The rotation of the γ subunit induces structural changes in the β subunit that result in the synthesis and release of ATP from the enzyme. Proton influx provides the force for the rotation.

The flow of two electrons through NADH-Q oxidoreductase, Q-cytochrome *c* oxidoreductase, and cytochrome *c* oxidase generates a gradient sufficient to synthesize 1, 0.5, and 1 molecule of ATP, respectively. Hence, 2.5 molecules of ATP are formed per molecule of NADH oxidized in the mitochondrial matrix, whereas only 1.5 molecules of ATP are made per molecule of $FADH_2$ oxidized because its electrons enter the chain at QH_2, after the first proton-pumping site.

Many Shuttles Allow Movement Across the Mitochondrial Membranes

Mitochondria employ a host of carriers, or transporters, to move molecules across the inner mitochondrial membrane. The electrons of cytoplasmic NADH are transferred into the mitochondria by the glycerol phosphate shuttle to form $FADH_2$ from FAD. The entry of ADP into the mitochondrial matrix is coupled to the exit of ATP by ATP-ADP translocase, a transporter driven by membrane potential.

The Regulation of Oxidative Phosphorylation Is Governed Primarily by the Need for ATP

About 30 molecules of ATP are generated when a molecule of glucose is completely oxidized to CO_2 and H_2O. Electron transport is normally tightly coupled to phosphorylation. NADH and $FADH_2$ are oxidized only if ADP is simultaneously phosphorylated to ATP, a form of regulation called acceptor or respiratory control. Uncouplers such as DNP can disrupt this coupling; they dissipate the proton gradient by carrying protons across the inner mitochondrial membrane. Proteins have been identified that uncouple electron transport and ATP synthesis for the generation of heat.

Key Terms

oxidative phosphorylation

proton-motive force

cellular respiration

electron-transport chain

reduction (redox, oxidation-reduction, E'_0) potential

inverted region

coenzyme Q (Q, ubiquinone)

NADH-Q oxidoreductase (Complex I)

flavin mononucleotide (FMN)

iron-sulfur (nonheme iron) protein

succinate-Q reductase (Complex II)

Q-cytochrome *c* oxidoreductase (Complex III)

cytochrome *c* (cyt *c*)

Rieske center

Q cycle

cytochrome *c* oxidase (Complex IV)

superoxide dismutase

catalase

ATP synthase (Complex V, F_1F_0 ATPase)

glycerol 3-phosphate shuttle

malate-aspartate shuttle

ATP-ADP translocase (adenine nucleotide translocase, ANT)

respiratory (acceptor) control

uncoupling protein (UCP)

programmed cell death (apoptosis)

caspase

Problems

1. *Energy harvest*. What is the yield of ATP when each of the following substrates is completely oxidized to CO_2 by a mammalian cell homogenate? Assume that glycolysis, the citric acid cycle, and oxidative phosphorylation are fully active.

   (a) Pyruvate

   (b) Lactate

   (c) Fructose 1,6-bisphosphate

   (d) Phosphoenolpyruvate

(e) Galactose

(f) Dihydroxyacetone phosphate

See answer

2. *Reference states*. The standard oxidation-reduction potential for the reduction of O_2 to H_2O is given as 0.82 V in Table 18.1. However, the value given in textbooks of chemistry is 1.23 V. Account for this difference.

See answer

3. *Potent poisons*. What is the effect of each of the following inhibitors on electron transport and ATP formation by the respiratory chain?

(a) Azide

(b) Atractyloside

(c) Rotenone

(d) DNP

(e) Carbon monoxide

(f) Antimycin A

See answer

4. *A question of coupling*. What is the mechanistic basis for the observation that the inhibitors of ATP synthase also lead to an inhibition of the electron-transport chain?

See answer

5. *O_2 consumption*. Using the axes in the adjoining illustration, draw an oxygen-uptake curve ($[O_2]$ versus time) for a suspension of isolated mitochondria when the following compounds are added in the indicated order. With the addition of each compound, all of the previously added compounds remain present. The experiment starts with the amount of oxygen indicated by the arrow on the *y*-axis. $[O_2]$ can only decrease or be unaffected.

(a) Glucose

(b) ADP + P_i

(c) Citrate

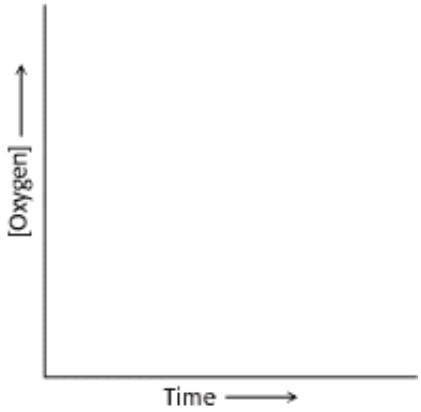
(d) Oligomycin

(e) Succinate

(f) Dinitrophenol

(g) Rotenone

(h) Cyanide



See answer

6. *P:O ratios.* The number of molecules of inorganic phosphate incorporated into organic form per atom of oxygen consumed, termed the *P:O ratio*, was frequently used as an index of oxidative phosphorylation.

(a) What is the relation of the P:O ratio to the ratio of the number of protons translocated per electron pair ($H^+/2\ e^-$) and the ratio of the number of protons needed to synthesize ATP and transport it to the cytosol (P/H^+)?

(b) What are the P:O ratios for electrons donated by matrix NADH and by succinate?

See answer

7. *Thermodynamic constraint.* Compare the $\Delta G^{\circ\prime}$ values for the oxidation of succinate by NAD^+ and by FAD. Use the data given in Table 18.1, and assume that E'_0 for the FAD-$FADH_2$ redox couple is nearly 0 V. Why is FAD rather than NAD^+ the electron acceptor in the reaction catalyzed by succinate dehydrogenase?

See answer

8. *Cyanide antidote.* The immediate administration of nitrite is a highly effective treatment for cyanide poisoning. What is the basis for the action of this antidote? (Hint: Nitrite oxidizes ferrohemoglobin to ferrihemoglobin.)

See answer

9. *Currency exchange*. For a proton-motive force of 0.2 V (matrix negative), what is the maximum $[ATP]/[ADP][P_i]$ ratio compatible with ATP synthesis? Calculate this ratio three times, assuming that the number of protons translocated per ATP formed is two, three, and four and that the temperature is 25°C.

See answer

10. *Runaway mitochondria 1*. Suppose that the mitochondria of a patient oxidizes NADH irrespective of whether ADP is present. The P:O ratio for oxidative phosphorylation by these mitochondria is less than normal. Predict the likely symptoms of this disorder.

See answer

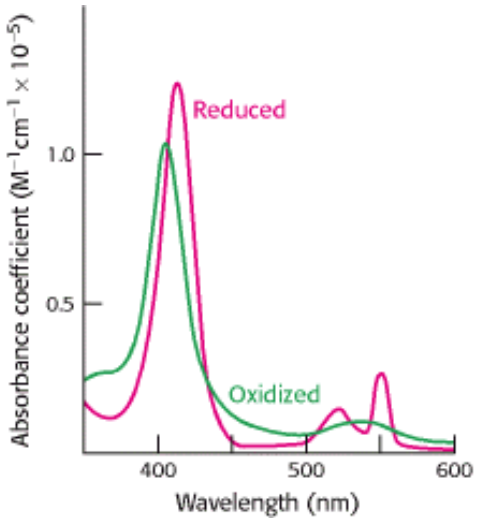
11. *An essential residue*. Conduction of protons by the F_0 unit of ATP synthase is blocked by modification of a single side chain by dicyclohexylcarbodiimide. What are the most likely targets of action of this reagent? How might you use site-specific mutagenesis to determine whether this residue is essential for proton conduction?

See answer

12. *Recycling device*. The cytochrome *b* component of Q-cytochrome *c* oxidoreductase enables both electrons of QH_2 to be effectively utilized in generating a proton-motive force. Cite another recycling device in metabolism that brings a potentially dead end reaction product back into the mainstream.

See answer

13. *Crossover point*. The precise site of action of a respiratory-chain inhibitor can be revealed by the *crossover technique*. Britton Chance devised elegant spectroscopic methods for determining the proportions of the oxidized and reduced forms of each carrier. This determiniation is feasible because the forms have distinctive absorption spectra, as illustrated in the adjoining graph for cytochrome *c*. You are given a new inhibitor and find that its addition to respiring mitochondria causes the carriers between NADH and QH_2 to become more reduced and those between cytochrome *c* and O_2 to become more oxidized. Where does your inhibitor act?



See answer

14. *Runaway mitochondria 2.* Years ago, it was suggested that uncouplers would make wonderful diet drugs. Explain why this idea was proposed and why it was rejected. Why might the producers of antiperspirants be supportive of the idea?

See answer

15. *Identifying the inhibition.* You are asked to determine whether a chemical is an electron-transport-chain inhibitor or an inhibitor of ATP synthase. Design an experiment to determine this.

See answer

Chapter Integration Problem

16. *No exchange.* Mice that are completely lacking adenine nucleotide translocase (ANT^{-}/ANT^{-}) can be made by the "knockout" technique. Remarkably, these mice are viable but have the following pathological conditions: (1) high serum levels of lactate, alanine, and succinate; (2) there is little electron transport; and (3) the levels of mitochondrial H_2O_2 are increased six- to eightfold compared with normal mice. Provide a possible biochemical explanation for each of these conditions.

See answer

Data Interpretation Problem

17. *Mitochondrial disease.* A mutation in a mitochondrial gene encoding a component of ATP synthase has been identified. People who have this mutation suffer from muscle weakness, ataxia, and retinitis pigmentosa. A tissue biopsy was performed on each patient, and submitochondrial particles were isolated that were capable of succinate-sustained ATP synthesis. First, the activity of the ATP synthase was measured on the addition of succinate and the following results were obtained.

	ATP synthase activity (nmol of ATP formed min^{-1} mg^{-1})
Controls	3.0
Patient 1	0.25
Patient 2	0.11
Patient 3	0.17

(a) What was the purpose of the addition of succinate?

(b) What is the effect of the mutation on succinate-coupled ATP synthesis?

Next, the ATPase activity of the enzyme was measured by incubating the submitochondrial particle with ATP in the absence of succinate.

	ATP hydrolysis (nmol of ATP hydrolyzed min^{-1} mg^{-1})
Controls	33
Patient 1	30
Patient 2	25
Patient 3	31

(c) Why was succinate omitted from the reaction?

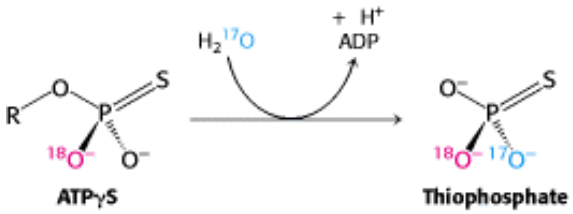
(d) What is the effect of the mutation on ATP hydrolysis?

(e) What do these results, in conjunction with those obtained in the first experiment, tell you about the nature of the mutation?

See answer

Mechanism Problem

18. *Chiral clue.* ATPγS, a slowly hydrolyzed analog of ATP, can be used to probe the mechanism of phosphoryl transfer reactions. Chiral ATPγS has been synthesized containing ^{18}O in a specific γ position and ordinary ^{16}O elsewhere in the molecule. Hydrolysis of this chiral molecule by ATP synthase in ^{17}O-enriched water yields inorganic [^{16}O,^{17}O,^{18}O]thiophosphate having the following absolute configuration. In contrast, hydrolysis of this chiral ATPγS by a calcium-pumping ATPase from muscle gives thiophosphate of the opposite configuration. What is the simplest interpretation of these data?



See answer

Media Problem

19. *Queueing up?* The **Conceptual Insights** module on oxidative phophorylation summarizes the steps in the electron transport chain and can help you answer the following questions. Suppose you have isolated actively respiring mitochondria. What do you think might happen to ATP production in the short-term if you were to add large amounts of coenzyme Q? If you were to add large amounts of QH_2? How might the long-term responses differ from the short-term?

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19. The Light Reactions of Photosynthesis

Essentially all free energy utilized by biological systems arises from solar energy that is trapped by the process of photosynthesis. The basic equation of photosynthesis is deceptively simple. Water and carbon dioxide combine to form carbohydrates and molecular oxygen.

$$CO_2 + H_2O \xrightarrow{\text{Light}} (CH_2O) + O_2$$

In this equation, (CH_2O) represents carbohydrate, primarily sucrose and starch. The mechanism of photosynthesis is complex and requires the interplay of many proteins and small molecules. Photosynthesis in green plants takes place in *chloroplasts* (Figure 19.1). *The energy of light captured by pigment molecules, called chlorophylls, in chloroplasts is*

used to generate high-energy electrons with great reducing potential. These electrons are used to produce NADPH as well as ATP in a series of reactions called the *light reactions* because they require light. NADPH and ATP formed by the action of light then reduce carbon dioxide and convert it into *3-phosphoglycerate* by a series of reactions called the *Calvin cycle* or the dark reactions. The Calvin cycle will be discussed in Chapter 20. The amount of energy stored by photosynthesis is enormous. More than 10^{17} kcal (4.2×10^{17} kJ) of free energy is stored annually by photosynthesis on Earth, which corresponds to the assimilation of more than 10^{10} tons of carbon into carbohydrate and other forms of organic matter.

As animals ourselves, we perhaps easily overlook the ultimate primacy of photosynthesis for our biosphere. Photosynthesis is the source of essentially all the carbon compounds and all the oxygen that makes aerobic metabolism possible. Moreover, as we shall see, there are considerable mechanistic and evolutionary parallels between the light reactions of photosynthesis and steps in oxidative phosphorylation.

19.0.1. Photosynthesis: An Overview

We can use our understanding of the citric acid cycle and oxidative phosphorylation to anticipate the processes required of photosynthesis. The citric acid cycle oxidizes carbon fuels to CO_2 to generate high-energy electrons, notably in the form of NADH. The flow of these high-energy electrons generates a proton-motive force through the action of the electron-transport chain. This proton-motive force is then transduced by ATP synthase to form ATP. A principal difference between oxidative phosphorylation and photosynthesis is the source of the high-energy electrons. The light reactions of photosynthesis use energy from *photons* to generate high-energy electrons (Figure 19.2). These electrons are used directly to reduce $NADP^+$ to NADPH and are used indirectly through an electron-transport chain to generate a proton-motive force across a membrane. A side product of these reactions is O_2. The proton-motive force drives ATP synthesis through the action of an ATP synthase, homologous to that in oxidative phosphorylation. In the dark reactions, the NADPH and ATP formed by the action of light drive the reduction of CO_2 to more-useful organic compounds.

Photosynthetic yield-

"If a year's yield of photosynthesis were amassed in the form of sugar cane, it would form a heap over two miles high and with a base 43 square miles."

-G. E. Fogge

If all of this sugar cane were converted into sugar cubes (0.5 inch on a side) and stacked end to end, the sugar cubes would extend 1.6×10^{10} miles, or to the planet Pluto.

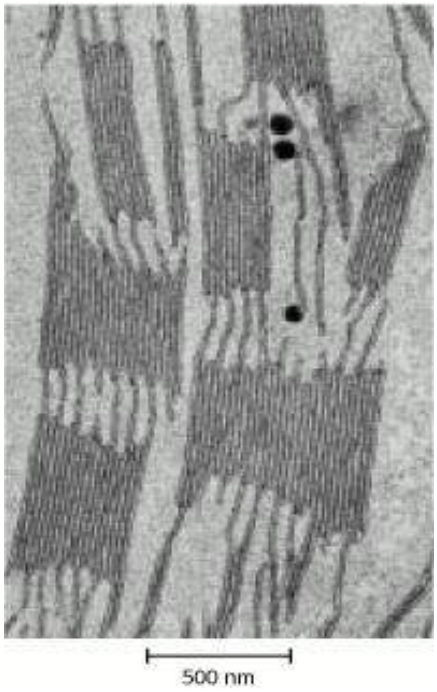


Figure 19.1. Electron Micrograph of a Chloroplast from a Spinach Leaf. The thylakoid membranes pack together to form grana. [Courtesy of Dr. Kenneth Miller.]

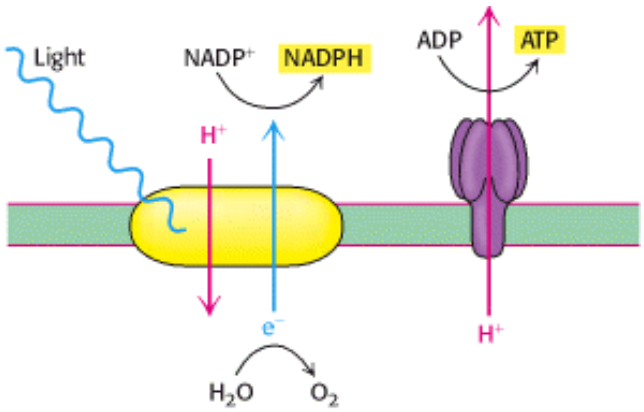
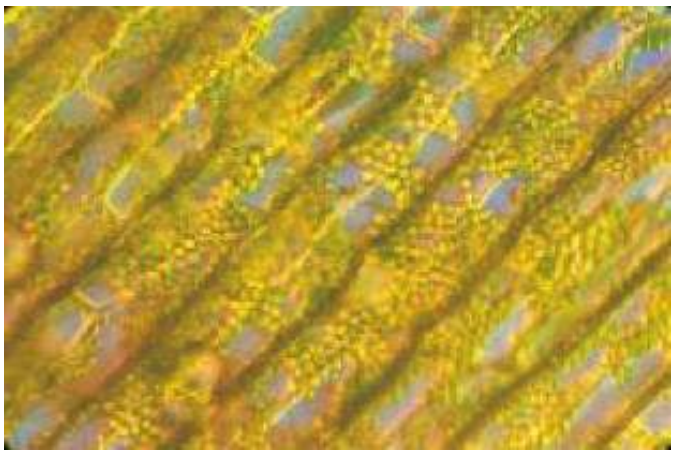
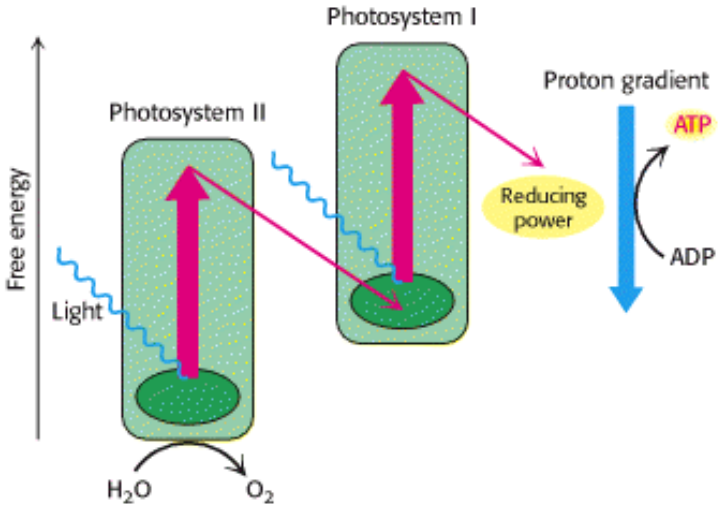


Figure 19.2. The Light Reactions of Photosynthesis. Light is absorbed and the energy is used to drive electrons from water to generate NADPH and to drive protons across a membrane. These protons return through ATP synthase to make ATP.





Chloroplasts (left) convert light energy into chemical energy. High-energy electrons in chloroplasts are transported through two photosystems (right). During this transit, which culminates in the generation of reducing power, ATP is synthesized in a manner analogous to mitochondrial ATP synthesis. Unlike as in mitochondrial electron transport, however, electrons in chloroplasts are energized by light. [(Left) Herb Charles Ohlmeyer/Fran Heyl Associates.]

19.1. Photosynthesis Takes Place in Chloroplasts

In Chapter 18, we saw that oxidative phosphorylation, the predominant means of generating ATP from fuel molecules, was compartmentalized into mitochondria. Likewise, photosynthesis, the means of converting light into chemical energy, is sequestered into organelles called *chloroplasts*, typically 5 µm long. Like a mitochondrion, a chloroplast has an outer membrane and an inner membrane, with an intervening intermembrane space (Figure 19.3). The inner membrane surrounds a *stroma*, which is the site of the carbon chemistry of photosynthesis (Section 20.1). In the stroma are membranous structures called *thylakoids*, which are flattened sacs, or discs. The thylakoid sacs are stacked to form a *granum*. Different grana are linked by regions of thylakoid membrane called *stroma lamellae*. The thylakoid membranes separate the thylakoid space from the stroma space. Thus, chloroplasts have three different membranes (*outer*, *inner*, and *thy-lakoid membranes*) and three separate spaces (*intermembrane*, *stroma*, and *thylakoid spaces*). In developing chloroplasts, thylakoids are believed to arise from invaginations of the inner membrane, and so they are analogous to the mitochondrial cristae. Like the mitochondrial cristae, they are the site of coupled oxidation-reduction reactions that generate the proton-motive force.

Photosynthetic catastrophe-

If photosynthesis were to cease, all higher forms of life would be extinct in about 25 years. A milder version of such a catastrophe ended the Cretaceous period 65.1 million years ago when a large asteroid struck the Yucatan Peninsula of Mexico. Enough dust was sent into the atmosphere that photosynthetic capacity was greatly diminished, which apparently led to the disappearance of the dinosaurs and allowed the mammals to rise to prominence.

19.1.1. The Primary Events of Photosynthesis Take Place in Thylakoid Membranes

The thylakoid membranes contain the energy-transducing machinery: light-harvesting proteins, reaction centers, electron-transport chains, and ATP synthase. They have nearly equal amounts of lipids and proteins. The lipid composition is highly distinctive: about 40% of the total lipids are *galactolipids* and 4% are *sulfolipids*, whereas only 10% are

phospholipids. The thylakoid membrane and the inner membrane, like the inner mitochondrial membrane, are impermeable to most molecules and ions. The outer membrane of a chloroplast, like that of a mitochondrion, is highly permeable to small molecules and ions. The stroma contains the soluble enzymes that utilize the NADPH and ATP synthesized by the thylakoids to convert CO_2 into sugar. Plant leaf cells contain between 1 and 100 chloroplasts, depending on the species, cell type, and growth conditions.

19.1.2. The Evolution of Chloroplasts

Chloroplasts contain their own DNA and the machinery for replicating and expressing it. However, chloroplasts are not autonomous: they also contain many proteins encoded by nuclear DNA. How did the intriguing relation between the cell and its chloroplasts develop? We now believe that, in a manner analogous to the evolution of mitochondria (Section 18.1.2), chloroplasts are the result of endosymbiotic events in which a photosynthetic microorganism, most likely an ancestor of a cyanobacterium (Figure 19.4), was engulfed by a eukaryotic host. Evidence suggests that chloroplasts in higher plants and green algae are derived from a single endosymbiotic event, whereas those in red and brown algae arose from at least one additional event.

The chloroplast genome is smaller than that of a cyanobacterium; however, like that of a cyanobacterium, it is circular with a single start site for DNA replication, and its genes are arranged in operons—sequences of functionally related genes under common control (Chapter 31). In the course of evolution, many of the genes of the chloroplast ancestor were transferred to the plant cell's nucleus or, in some cases, lost entirely, thus establishing a fully dependent relation.

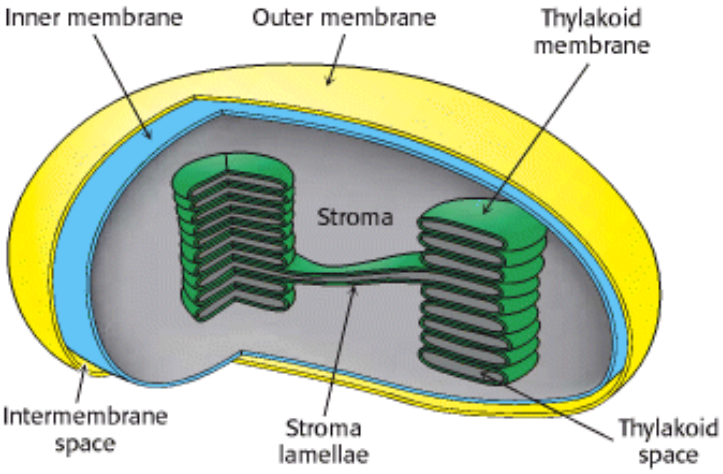


Figure 19.3. Diagram of a Chloroplast. [After S. L. Wolfe, *Biology of the Cell*, p. 130. © 1972 by Wadsworth Publishing Company, Inc. Adapted by permission of the publisher.]

Figure 19.4. Cyanobacteria. A colony of the photosynthetic filamentous cyanobacteria *Anabaena* shown at 450× magnification. Ancestors of these bacteria are thought to have evolved into present-day chloroplasts. [James W. Richardson/Visuals Unlimited.]

19.2. Light Absorption by Chlorophyll Induces Electron Transfer

The trapping of light energy is the key to photosynthesis. The first event is the absorption of light by a photoreceptor molecule. The principal photoreceptor in the chloroplasts of most green plants is *chlorophyll* a, a substituted tetrapyrrole (Figure 19.5). The four nitrogen atoms of the pyrroles are coordinated to a magnesium ion. Unlike a porphyrin such as heme, chlorophyll has a reduced pyrrole ring. Another distinctive feature of chlorophyll is the presence of *phytol*, a highly hydrophobic 20-carbon alcohol, esterified to an acid side chain.

Chlorophylls are very effective photoreceptors because they contain networks of alternating single and double bonds. Such compounds are called *polyenes*. They have very strong absorption bands in the visible region of the spectrum, where the solar output reaching Earth also is maximal (Figure 19.6). The peak molar absorption coefficient (ε, Section 3.1) of chlorophyll *a* is higher than 10^5 M^{-1} cm^{-1}, among the highest observed for organic compounds.

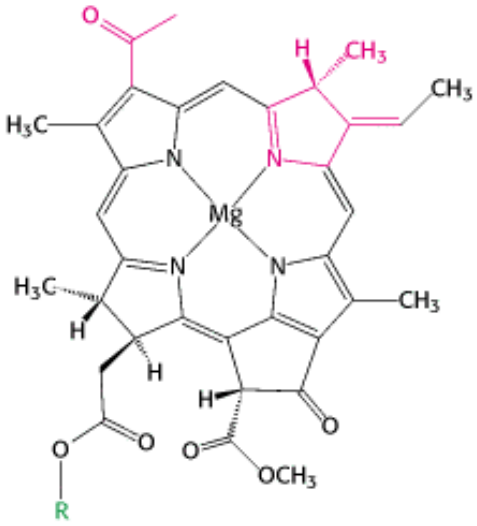
What happens when light is absorbed by a molecule such as chlorophyll? The energy from the light excites an electron from its ground energy level to an excited energy level (Figure 19.7). This high-energy electron can have several fates. For most compounds that absorb light, the electron simply returns to the ground state and the absorbed energy is converted into heat. However, if a suitable electron acceptor is nearby, the excited electron can move from the initial molecule to the acceptor (Figure 19.8). This process results in the formation of a positive charge on the initial molecule (due to the loss of an electron) and a negative charge on the acceptor and is, hence, referred to as *photoinduced charge separation*. The site where the separational change occurs is called the *reaction center*. We shall see how the photosynthetic apparatus is arranged to make photoinduced charge separation extremely efficient. The electron, extracted from its initial site by absorption of light, can reduce other species to store the light energy in chemical forms.

19.2.1. Photosynthetic Bacteria and the Photosynthetic Reaction Centers of Green Plants Have a Common Core

Photosynthesis in green plants is mediated by two kinds of membrane-bound, light-sensitive complexes— *photosystem I (PS I)* and *photosystem II (PS II)*. Photosystem I typically includes 13 polypeptide chains, more than 60 chlorophyll molecules, a quinone (vitamin K_1), and three 4Fe-4S clusters. The total molecular mass is more than 800 kd. Photosystem II is only slightly less complex with at least 10 polypeptide chains, more than 30 chlorophyll molecules, a nonheme iron ion, and four manganese ions. Photosynthetic bacteria such as *Rhodopseudomonas viridis* contain a simpler, single type of photosynthetic reaction center, the structure of which was revealed at atomic resolution. The bacterial reaction center consists of four polypeptides: L (31 kd), M (36 kd), and H (28 kd) subunits and C, a *c*-type cytochrome (Figure 19.9). *The results of sequence comparisons and low-resolution structural studies of photosystems I and II revealed that the bacterial reaction center is homologous to the more complex plant systems*. Thus, we begin our consideration of the mechanisms of the light reactions within the bacterial photosynthetic reaction center, with the understanding that many of our observations will apply to the plant systems as well.

19.2.2. A Special Pair of Chlorophylls Initiates Charge Separation

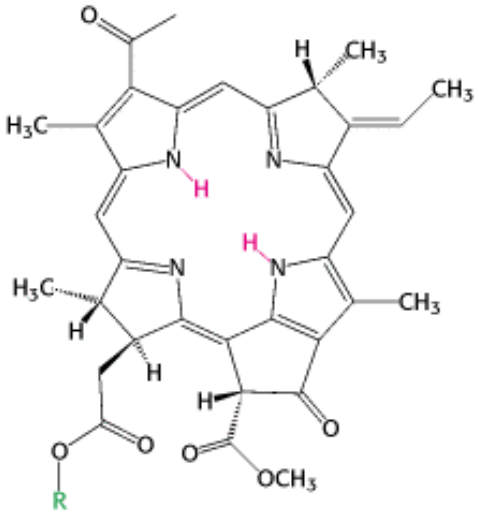
The L and M subunits form the structural and functional core of the bacterial photosynthetic reaction center (see Figure 19.9). Each of these homologous subunits contains five transmembrane helices. The H subunit, which has only one transmembrane helix, lies on the cytoplasmic side of the membrane. The cytochrome subunit, which contains four *c*-type hemes, lies on the opposite periplasmic side. Four bacteriochlorophyll *b* (BChl-*b*) molecules, two bacteriopheophytin *b* (BPh) molecules, two quinones (Q_A and Q_B), and a ferrous ion are associated with the L and M subunits.



Bacteriochlorophyll *b*
(BChl-*b*)

Bacteriochlorophylls are similar to chlorophylls, except for the reduction of an additional pyrrole ring and some other minor differences that shift their absorption maxima to the near infrared, to wavelengths as long as 1000 nm. *Bacteriopheophytin* is the term for a bacteriochlorophyll that has two protons instead of a magnesium ion at its center.

The reaction begins with light absorption by a dimer of BChl-*b* molecules that lie near the periplasmic side of the membrane. This dimer, called a *special pair* because of its fundamental role in photosynthesis, absorbs light maximally at 960 nm, in the infrared near the edge of the visible region. For this reason, the special pair is often referred to as *P960* (P stands for pigment). Excitation of the special pair leads to the ejection of an electron, which is transferred through another molecule of BChl-*b* to the bacteriopheophytin in the L subunit (Figure 19.10, steps 1 and 2). This initial charge separation, which yields a positive charge on the special pair ($P960^+$) and a negative charge on BPh, occurs in less than 10 picoseconds (10^{-11} seconds). Interestingly, only one of the two possible paths within the nearly symmetric L-M dimer is utilized. In their high-energy states, $P960^+$ and BPh^- could undergo charge recombination; that is, the electron on BPh^- could move back to neutralize the positive charge on the special pair. Its return to the special pair would waste a valuable high-energy electron and simply convert the absorbed light energy into heat. Three factors in the structure of the reaction center work together to suppress charge recombination nearly completely (Figure 19.10, steps 3 and 4). First, another electron acceptor, a tightly bound quinone (Q_A), is less than 10 Å away from BPh^-, and so the electron is rapidly transferred farther away from the special pair. Recall that electron-transfer rates depend strongly on distance (Section 18.2.3). Second, one of the hemes of the cytochrome subunit is less than 10 Å away from the special pair, and so the positive charge is neutralized by the transfer of an electron from the reduced cytochrome. Finally, the electron transfer from BPh^- to the positively charged special pair is especially slow: the transfer is so thermodynamically favorable that it takes place in the inverted region where electron-transfer rates become slower (Section 18.2.3). Thus, electron transfer proceeds efficiently from BPh^- to Q_A.



From Q_A, the electron moves to a more loosely associated quinone, Q_B. The absorption of a second photon and the movement of a second electron down the path from the special pair completes the two-electron reduction of Q_B from Q to QH_2. Because the Q_B-binding site lies near the cytoplasmic side of the membrane, two protons are taken up from the cytoplasm, contributing to the development of a proton gradient across the cell membrane (Figure 19.10, steps 5, 6, and 7).

How does the cytochrome subunit of the reaction center regain an electron to complete the cycle? The reduced quinone (QH_2) is reoxidized to Q by complex III of the respiratory electron-transport chain (Section 18.3.3). The electrons from the reduced quinone are transferred through a soluble cytochrome *c* intermediate, called cytochrome c_2, in the periplasm to the cytochrome subunit of the reaction center. The flow of electrons is thus cyclic. The proton gradient generated in the course of this cycle drives the generation of ATP through the action of ATP synthase.

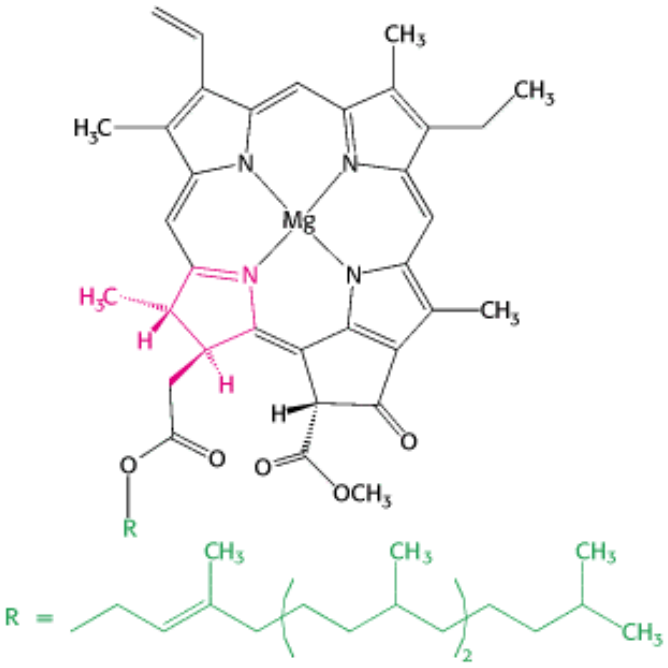


Figure 19.5. Chlorophyll. Like heme, chlorophyll *a* is a cyclic tetrapyrrole. One of the pyrrole rings (shown in red) is reduced. A phytol chain (shown in green) is connected by an ester linkage. Magnesium ion binds at the center of the structure.

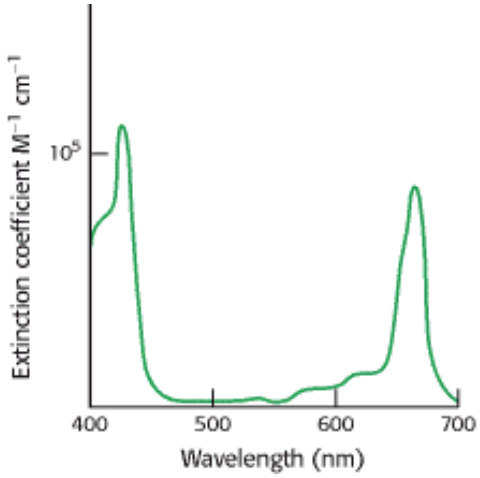


Figure 19.6. Light Absorption By Chlorophyll *A*. Chlorophyll *a* absorbs visible light efficiently as judged by the extinction coefficients near 10^5 M^{-1} cm^{-1}.

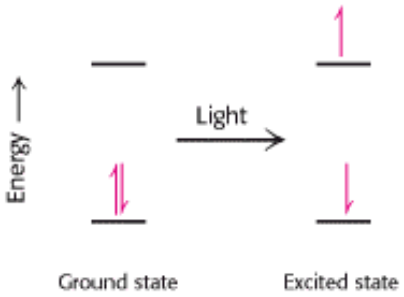


Figure 19.7. Light Absorption. The absorption of light leads to the excitation of an electron from its ground state to a higher energy level.

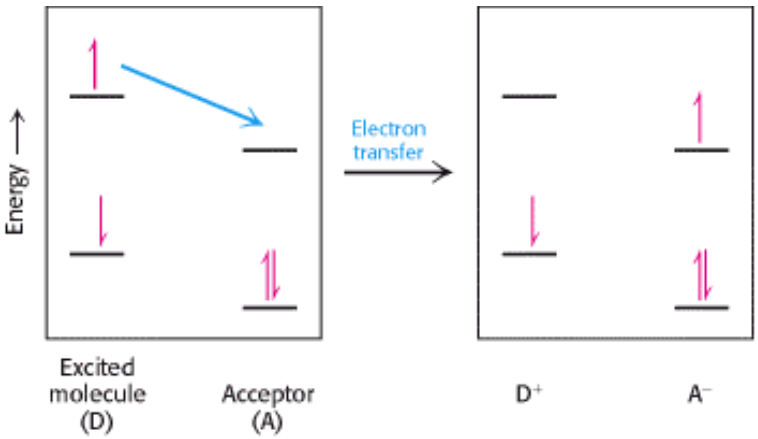


Figure 19.8. Photoinduced Charge Separation. If a suitable electron acceptor is nearby, an electron that has been moved to a high energy level by light absorption can move from the excited molecule to the acceptor.

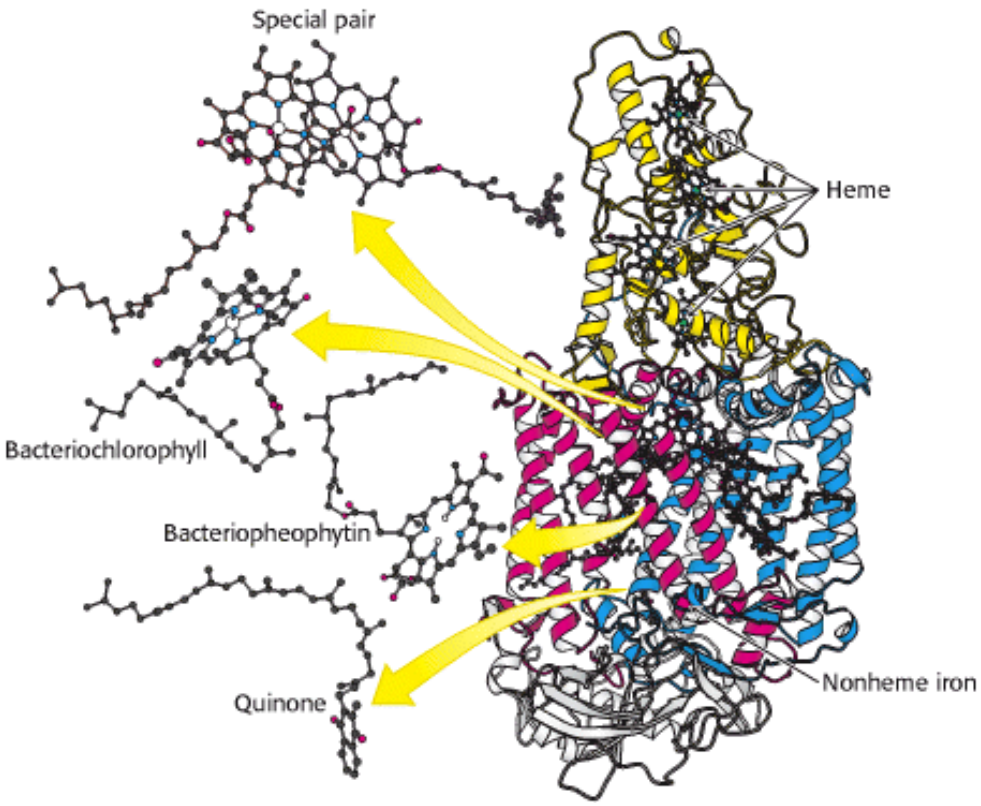


Figure 19.9. Bacterial Photosynthetic Reaction Center. The core of the reaction center from *Rhodopseudomonas viridis* consists of two similar chains: L (red) and M (blue). An H chain (white) and a cytochrome subunit (yellow) complete the structure. A chain of prosthetic groups, beginning with a special pair of bacteriochlorophylls and ending at a bound quinone, runs through the structure from top to bottom in this view.

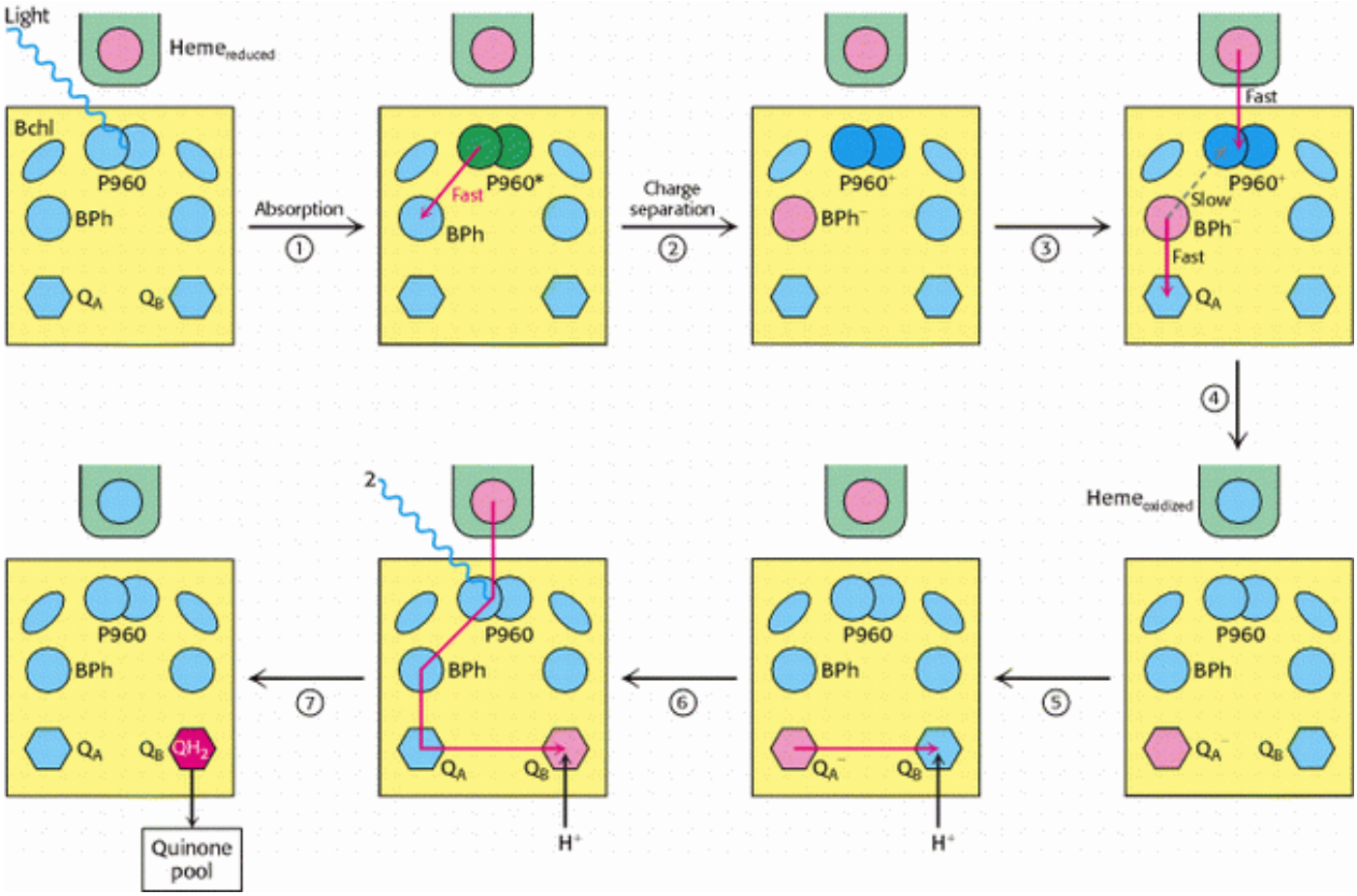


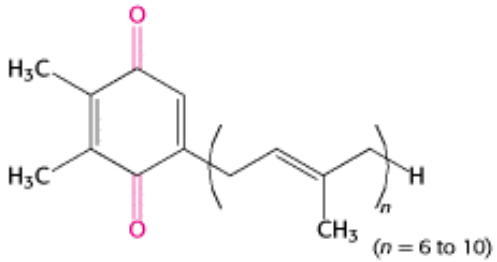
Figure 19.10. Electron Chain in the Photosynthetic Bacterial Reaction Center. The absorption of light by the special pair (P960) results in the rapid transfer of an electron from this site to a bacteriopheophytin (BPh), creating a photoinduced charge separation (steps 1 and 2). (The asterisk on P960 stands for excited state.) The possible return of the electron from the pheophytin to the oxidized special pair is suppressed by the "hole" in the special pair being refilled with an electron from the cytochrome subunit and the electron from the pheophytin being transferred to a quinone (Q_A) that is farther away from the special pair (steps 3 and 4). The reduction of a quinone (Q_B) on the periplasmic side of the membrane results in the uptake of two protons from the periplasmic space (steps 5 and 6). The reduced quinone can move into the quinone pool in the membrane (step 7).

19.3. Two Photosystems Generate a Proton Gradient and NADPH in Oxygenic Photosynthesis

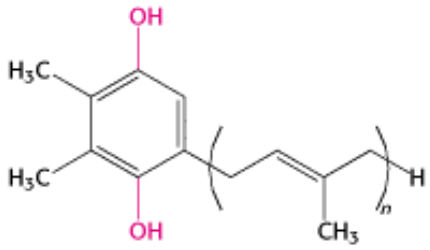
Photosynthesis by oxygen-evolving organisms depends on the interplay of two photosystems, linked by common intermediates (Figure 19.11). These two systems were discovered because of slight differences in the wavelengths of light to which they respond. Photosystem I responds to light with wavelengths shorter than 700 nm, whereas photosystem II responds to wavelengths shorter than 680 nm. Under normal conditions, electrons flow first through photosystem II, then through cytochrome *bf*, a membrane-bound complex homologous to Q-cytochrome *c* oxidoreductase from oxidative phosphorylation (Section 18.3.3), and then through photosystem I. The electrons are derived from water: two molecules of H_2O are oxidized to generate a molecule of O_2 for every four electrons sent through this electron-transport chain. The electrons end up reducing $NADP^+$ to NADPH, a versatile reagent for driving biosynthetic processes. These processes generate a proton gradient across the thylakoid membrane that drives the formation of ATP.

19.3.1. Photosystem II Transfers Electrons from Water to Plastoquinone and Generates a Proton Gradient

Photosystem II of green plants is reasonably similar to the bacterial reaction center (Figure 19.12). The core of photosystem II is formed by D1 and D2, a pair of similar 32-kd subunits that span the thylakoid membrane. These subunits are homologous to the L and M chains of the bacterial reaction center. Unlike the bacterial system, photosystem II contains a large number of additional subunits that bind additional chlorophylls and increase the efficiency with which light energy is absorbed and transferred to the reaction center (Section 19.5).



Plastoquinone
(oxidized form, Q)



Plastoquinol
(reduced form, QH_2)

The overall reaction catalyzed by photosystem II is:

$$2\,Q + 2\,H_2O \xrightarrow{\text{Light}} O_2 + 2\,QH_2$$

in which Q represents plastoquinone and QH_2 represents plastoquinol. This reaction is similar to one catalyzed by the bacterial system in that a quinone is converted from its oxidized into its reduced form. However, instead of obtaining the electrons for this reduction from a reduced cytochrome *c* molecule, photosystem II extracts the electrons from water, generating molecular oxygen. This remarkable reaction takes place at a special center containing four manganese ions.

The photochemistry of photosystem II begins with excitation of a special pair of chlorophyll molecules that are bound by the D1 and D2 subunits (Figure 19.13). This pair of molecules is analogous to the special pair in the bacterial reaction center, but it absorbs light at shorter wavelengths (maximum absorbance at 680 nm) because it consists of chlorophyll *a* molecules rather than bacteriochlorophyll. The special pair is often called *P680*. On excitation, P680 rapidly transfers an electron to a nearby pheophytin (chlorophyll with two H^+ ions in place of the central Mg^{2+} ion). From there, the electron is transferred first to a tightly bound plastoquinone at site Q_A and then to an exchangeable plastoquinone at site Q_B. This electron flow is entirely analogous to that in the bacterial system. With the arrival of a second electron and the uptake of two protons, the exchangeable plastoquinone is reduced to QH_2.

The major difference between the bacterial system and photosystem II is the source of the electrons that are used to neutralize the positive charge formed on the special pair. *$P680^+$, a very strong oxidant, extracts electrons from water molecules bound at the manganese center*. The structure of this center, which includes four manganese ions, a calcium ion, a chloride ion, and a tyrosine residue that forms a radical, has not been fully established, although the results of extensive spectroscopic studies and a recent X-ray crystallographic study at moderate resolution have provided many constraints. Manganese was apparently evolutionarily selected for this role because of its ability to exist in multiple

oxidation states (Mn^{2+}, Mn^{3+}, Mn^{4+}, Mn^{5+}) and to form strong bonds with oxygen-containing species. The manganese center, in its reduced form, oxidizes two molecules of water to form a single molecule of oxygen. Each time the absorbance of a photon kicks an electron out of P680, the positively charged special pair extracts an electron from the manganese center (Figure 19.14). Thus four photochemical steps are required to extract the electrons and reduce the manganese center (Figure 19.15). The four electrons harvested from water are used to reduce two molecules of Q to QH_2.

Photosystem II spans the thylakoid membrane such that the site of quinone reduction is on the side of the stroma, whereas the manganese center and, hence, the site of water oxidation lies in the thylakoid lumen. Thus, the two protons that are taken up with the reduction of each molecule of plastoquinone come from the stroma, and the four protons that are liberated in the course of water oxidation are released into the lumen. This distribution of protons generates a proton gradient across the thylakoid membrane characterized by an excess of protons in the thylakoid lumen compared with the stroma (Figure 19.16). Thus, the direction of the proton gradient is the reverse of that generated during oxidative phosphorylation, which depletes the mitochondrial matrix of protons. As we shall see, this difference is consistent with the reversed orientations of other membrane proteins, including ATP synthase.

19.3.2. Cytochrome *bf* Links Photosystem II to Photosystem I

The plastoquinol (QH_2) produced by photosystem II contributes its electrons to continue the electron chain that terminates at photosystem I. These electrons are transferred, one at a time, to plastocyanin (Pc), a copper protein in the thylakoid lumen.

$$QH_2 + 2\,Pc(Cu^{2+}) \longrightarrow Q + 2\,Pc(Cu^{+}) + 2\,H^{+}_{\text{thylakoid lumen}}$$

The two protons from plastoquinol are released into the thylakoid lumen. This reaction is reminiscent of that catalyzed by ubiquinol cytochrome *c* oxidoreductase in oxidative phosphorylation. Indeed, most components of the enzyme complex that catalyzes the reaction, the *cytochrome* bf complex, are homologous to those of ubiquinol cytochrome *c* oxidoreductase. The cytochrome *bf* complex includes four subunits: a 23-kd cytochrome with two *b*-type hemes, a 20-kd Rieske-type Fe-S protein, a 33-kd cytochrome *f* with a *c*-type cytochrome, and a 17-kd chain.

This complex catalyzes the reaction through the Q cycle (Section 18.3.4). In the first half of the Q cycle, plastoquinol is oxidized to plastoquinone, one electron at a time. The electrons from plastoquinol flow through the Fe-S protein to convert oxidized plastocyanin into its reduced form. Plastocyanin is a small, soluble protein with a single copper ion bound by a cysteine residue, two histidine residues, and a methionine residue in a distorted tetrahedral arrangement (Figure 19.17). This geometry facilitates the interconversion between the Cu^{2+} and the Cu^{+} states and sets the reduction potential at an appropriate value relative to that of plastoquinol. Plastocyanin is intensely blue in color in its oxidized form, marking it as a member of the "blue copper protein," or type I copper protein family.

The oxidation of plastoquinol results in the release of two protons into the thylakoid lumen. In the second half of the Q cycle (Section 18.3.4), cytochrome *bf* reduces a second molecule of plastoquinone from the Q pool to plastoquinol, taking up two protons from one side of the membrane, and then reoxidizes plastoquinol to release these protons on the other side. The enzyme is oriented so that protons are released into the thylakoid lumen and taken up from the stroma, contributing further to the proton gradient across the thylakoid membrane (Figure 19.18).

19.3.3. Photosystem I Uses Light Energy to Generate Reduced Ferredoxin, a Powerful Reductant

The final stage of the light reactions is catalyzed by photosystem I (Figure 19.19). The core of this system is a pair of similar subunits psaA (83 kd) and psaB (82 kd). These subunits are quite a bit larger than the core subunits of photosystem II and the bacterial reaction center. Nonetheless, they appear to be homologous; the terminal 40% of each subunit is similar to a corresponding subunit of photosytem II. A special pair of chlorophyll *a* molecules lies at the center

of the structure and absorb light maximally at 700 nm. This center, *P700*, initiates photoinduced charge separation (Figure 19.20). The electron is transferred down a pathway through chlorophyll at site A_0 and quinone at site A_1 to a set of 4Fe-4S clusters. From there, the electron is transferred to ferredoxin (Fd), a soluble protein containing a 2Fe-2S cluster coordinated to four cysteine residues (Figure 19.21). The positive charge of $P700^+$ is neutralized by the transfer of an electron from reduced plastocyanin. Thus, the overall reaction catalyzed by photosystem I is a simple one-electron oxidation-reduction reaction.

$$Pc(Cu^+) + Fd_{ox} \xrightarrow{\text{Light}} Pc(Cu^{2+}) + Fd_{red}$$

Given that the reduction potentials for plastocyanin and ferredoxin are +0.37 V and -0.45 V, respectively, the standard free energy for the oxidation of reduced plastocyanin by oxidized ferredoxin is +18.9 kcal mol^{-1} (+79.1 kJ mol^{-1}). This uphill reaction is driven by the absorption of a 700-nm photon which has an energy of 40.9 kcal mol^{-1} (171 kJ mol^{-1}).

The cooperation between photosystem I and photosystem II creates electron flow from H_2O to $NADP^+$. The pathway of electron flow is called the *Z scheme of photosynthesis* because the redox diagram from P680 to P700* looks like the letter Z (Figure 19.22).

19.3.4. Ferredoxin-$NADP^+$ Reductase Converts $NADP^+$ into NADPH

Although reduced ferredoxin is a strong reductant, it is not useful for driving many reactions, in part because ferredoxin carries only one available electron. In contrast, NADPH, a two-electron reductant, is widely used in biosynthetic processes, including the reactions of the Calvin cycle (Chapter 20). How can reduced ferredoxin be used to drive the reduction of $NADP^+$ to NADPH? This reaction is catalyzed by *ferredoxin-NADP $^+$ reductase*, a flavoprotein (Figure 19.23A). The bound FAD moiety in this enzyme accepts electrons, one at a time, from two molecules of reduced ferredoxin as it proceeds from its oxidized form, through a semiquinone intermediate, to its fully reduced form (Figure 19.23B). The enzyme then transfers a hydride ion to $NADP^+$ to form NADPH. Note that this reaction takes place on the stromal side of the membrane. Hence, the uptake of a proton in the reduction of $NADP^+$ further contributes to the proton gradient across the thylakoid membrane.

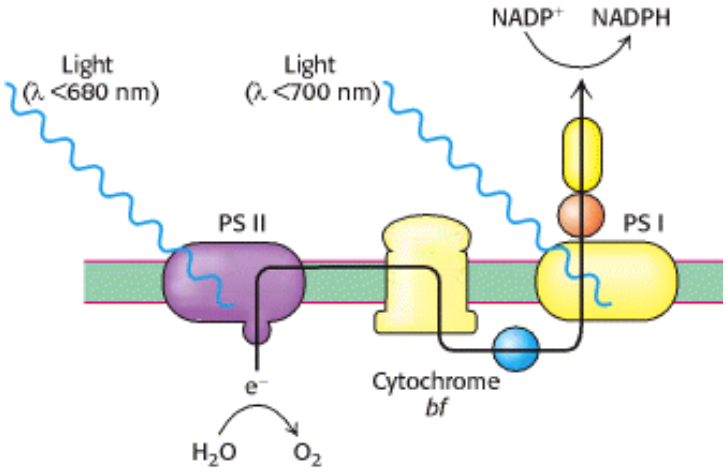


Figure 19.11. Two Photosystems. The absorption of photons by two distinct photosytems (PS I and PS II) is required for complete electron flow from water to $NADP^+$.

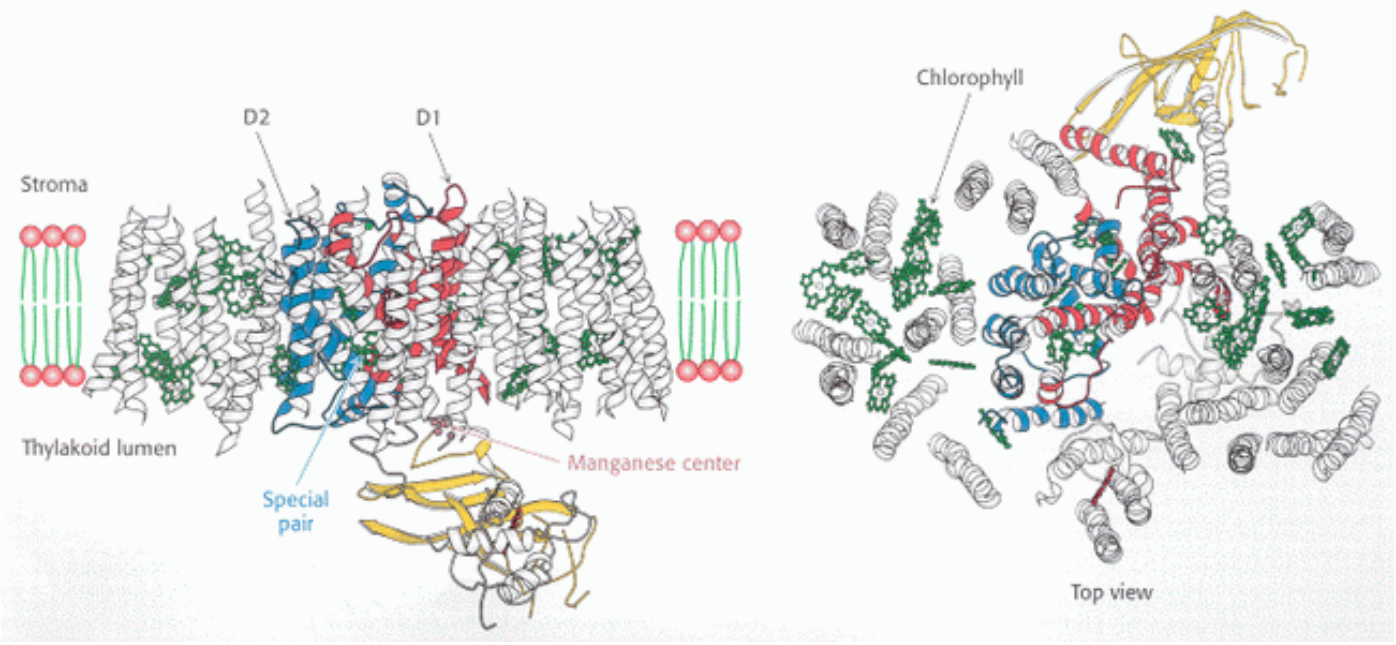


Figure 19.12. The Structure of Photosystem II. The D1 and D2 subunits are shown in red and blue and the structure of a bound cytochrome molecule is shown in yellow. Chlorophyll molecules are shown in green.

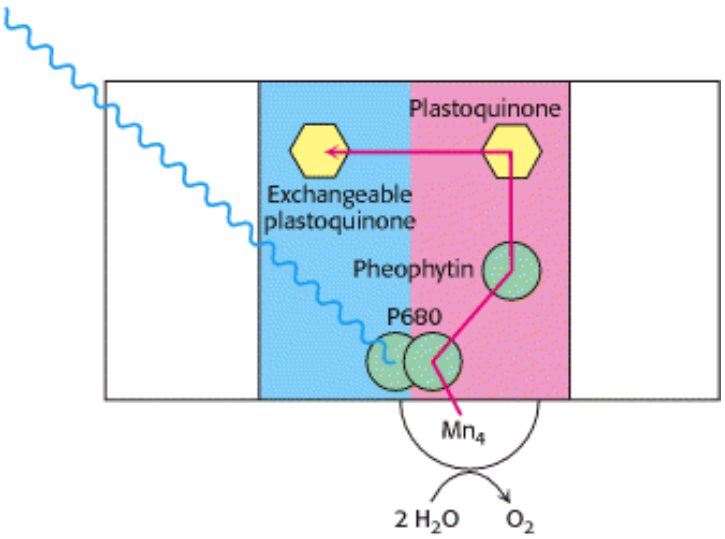


Figure 19.13. Electron Flow Through Photosystem II. Light absorption induces electron transfer from P680 down an electron-transfer pathway to an exchangeable plastoquinone. The positive charge on P680 is neutralized by electron flow from water molecules bound at the manganese center.

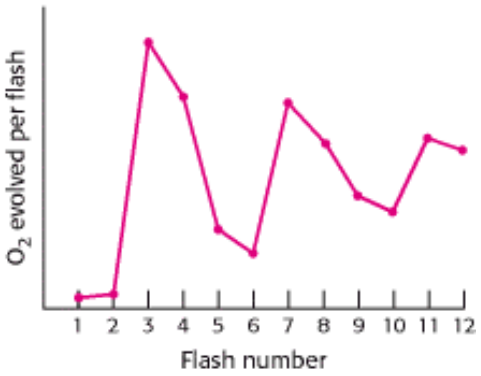


Figure 19.14. Four Photons Are Required to Generate One Oxygen Molecule. When dark-adapted chloroplasts are exposed to a brief flash of light, one electron passes through photosystem II. Monitoring the O_2 released after each flash reveals that four flashes are required to generate each O_2 molecule. The peaks in O_2 release occur after the 3rd, 7th, and 11th flashes because the dark-adapted chloroplasts start in the S_1 state-that is, the one-electron reduced state.

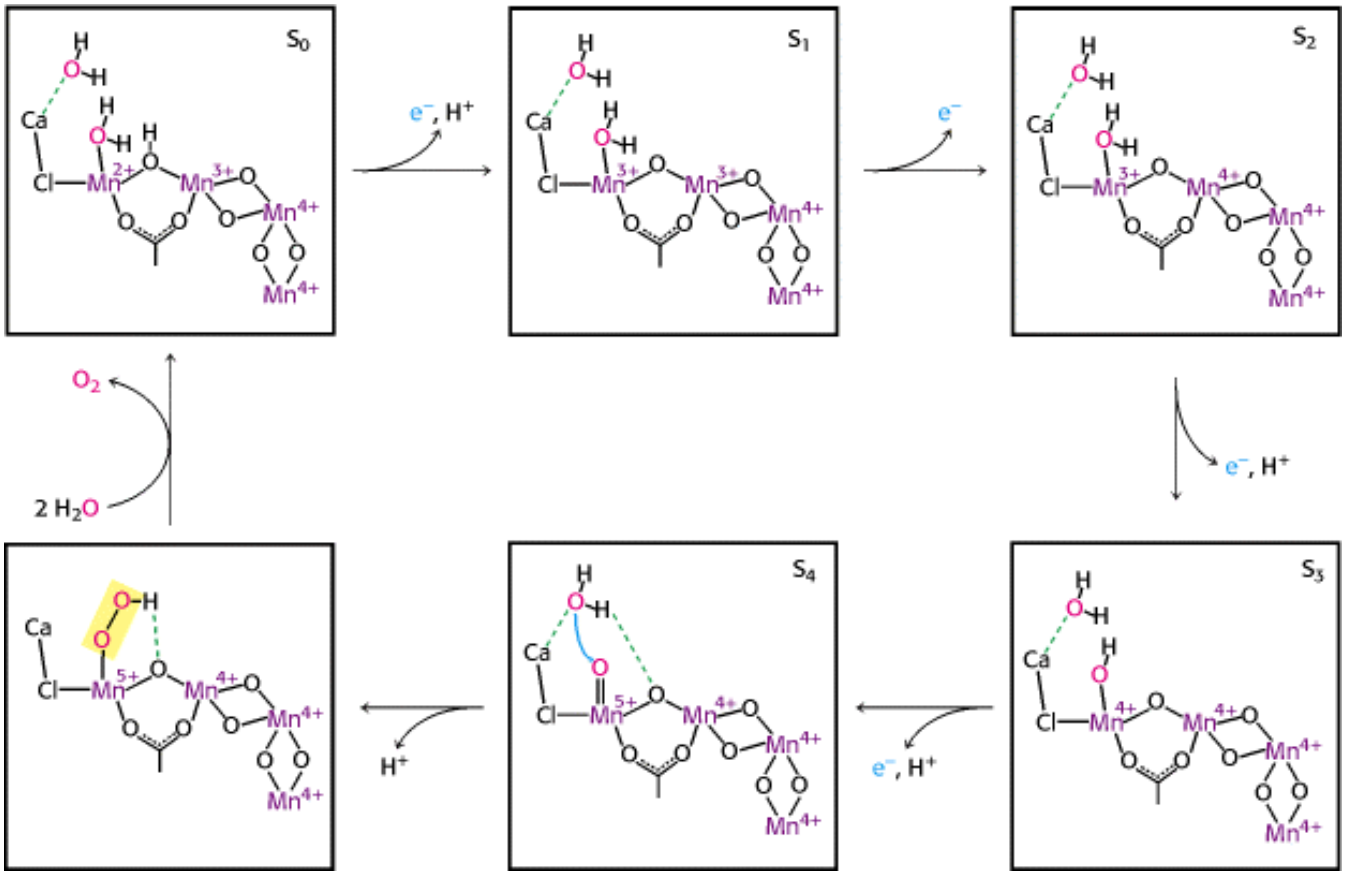


Figure 19.15. A Plausible Scheme for Oxygen Evolution from the Manganese Center. A possible partial structure for the manganese center is shown. The center is oxidized, one electron at a time, until two bound H_2O molecules are linked to form a molecule of O_2, which is then released from the center. A tyrosine residue (not shown) also participates in the coupled proton-electron transfer steps. The structures are designated S_0 through S_4 to indicate the number of electrons that have been removed.

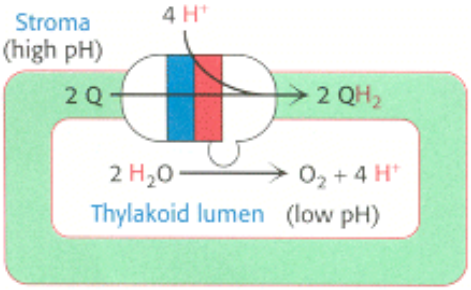


Figure 19.16. Proton-Gradient Direction. Photosystem II releases protons into the thylakoid lumen and takes them up from the stroma. The result is a pH gradient across the thylakoid membrane with an excess of protons (low pH) inside.

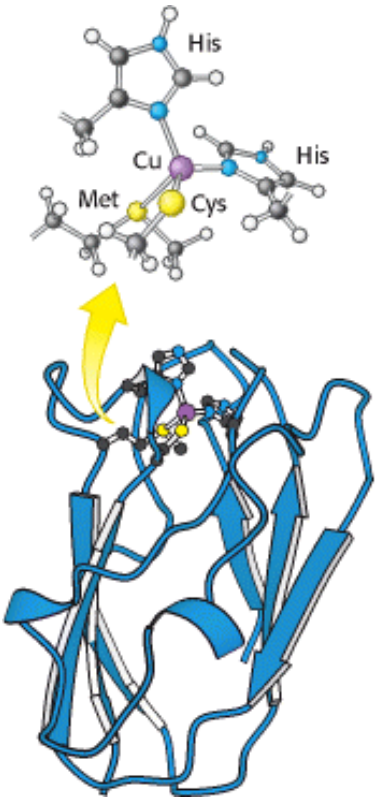


Figure 19.17. Structure of Plastocyanin. Two histidine residues, a cysteine residue, and a methionine residue coordinate a copper ion in a distorted tetrahedral manner in this protein, which carries electrons from cytochrome *bf* complex to photosystem I.

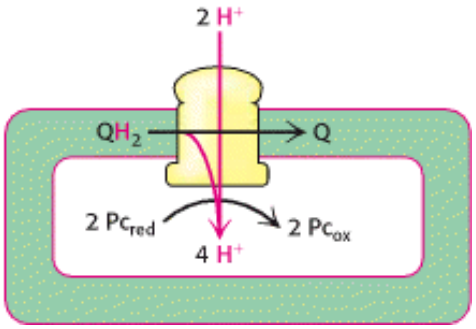


Figure 19.18. Cytochrome *BF* Contribution to Proton Gradient. The cytochrome *bf* complex oxidizes QH_2 to Q

through the Q cycle. Four protons are released into the thylakoid lumen in each cycle.

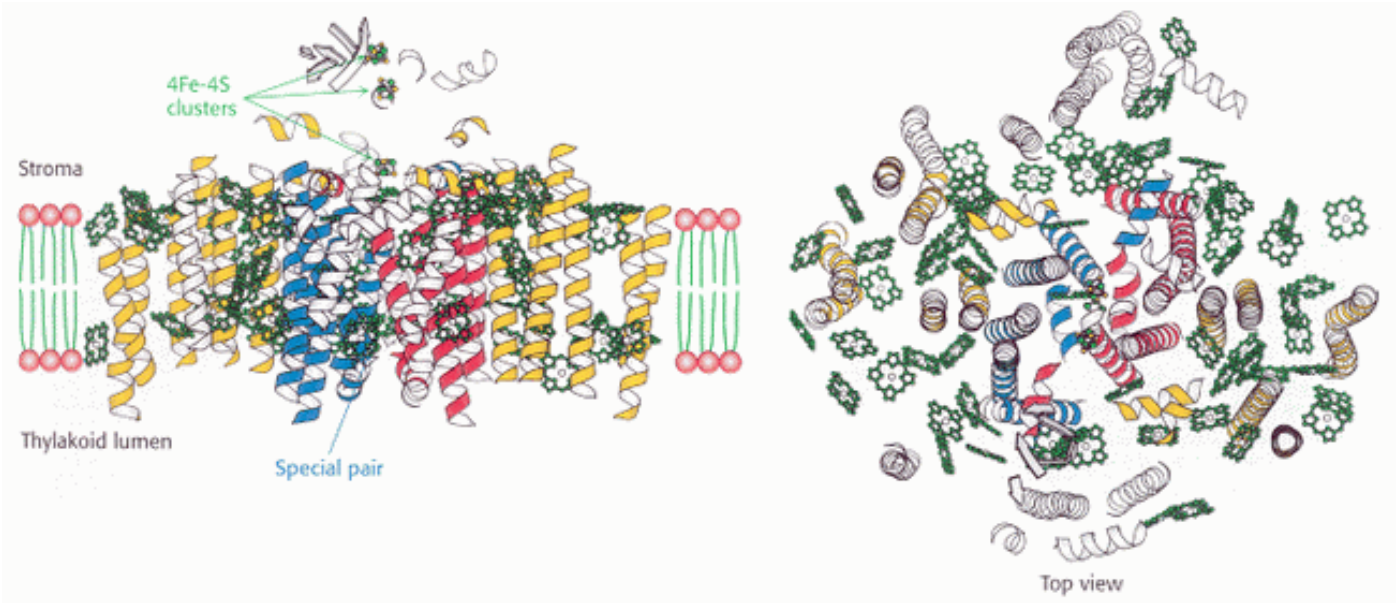


Figure 19.19. Structure of Photosystem I. The psaA and psaB subunits are shown in yellow, with the regions similar to those in the core of photosytem II shown in red and blue. Chlorophyll molecules are shown in green and three 4Fe-4S clusters are indicated.

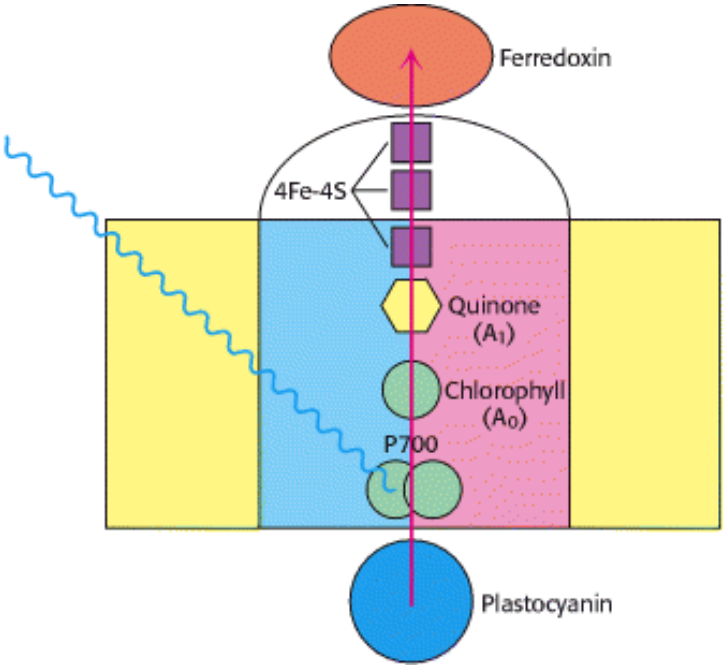


Figure 19.20. Electron Flow Through Photosystem I to Ferredoxin. Light absorption induces electron transfer from P700 down an electron-transfer pathway that includes a chlorophyll molecule, a quinone molecule, and three 4Fe-4S clusters to reach ferredoxin. The positive charge left on P700 is neutralized by electron transfer from reduced plastocyanin.

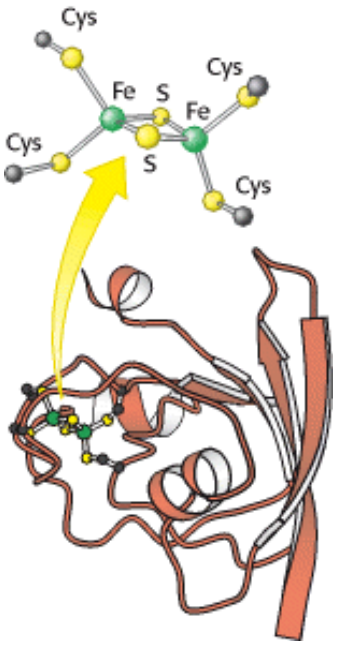


Figure 19.21. Structure of Ferredoxin. In plants, ferredoxin contains a 2Fe-2S cluster. This protein accepts electrons from photosystem I and carries them to ferredoxin-NADP$^+$ reductase.

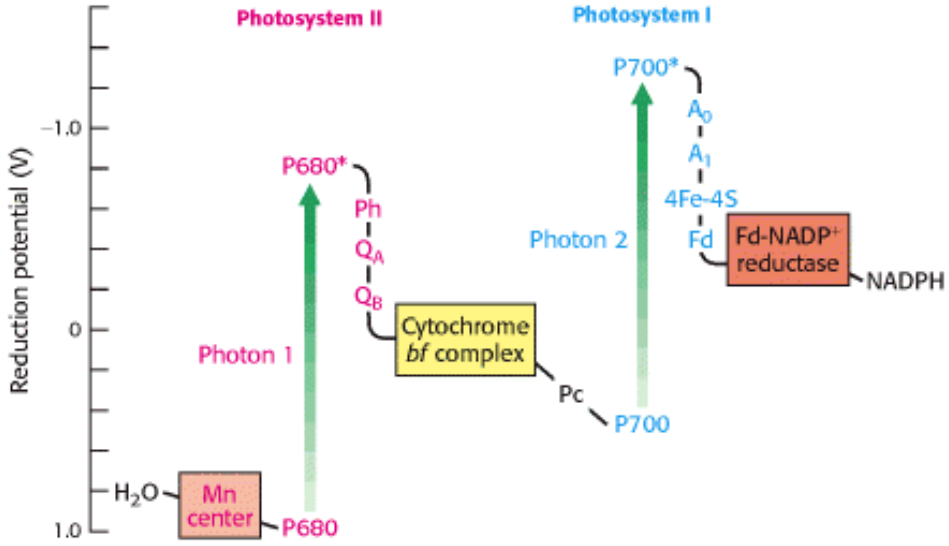


Figure 19.22. Pathway of Electron Flow From H_2O to NADP$^+$ in Photosynthesis. This endergonic reaction is made possible by the absorption of light by photosystem II (P680) and photosystem I (P700). Abbreviations: Ph, pheophytin; Q_A and Q_B, plastoquinone-binding proteins; Pc, plastocyanin; A_0 and A_1, acceptors of electrons from P700*; Fd, ferredoxin; Mn, manganese.

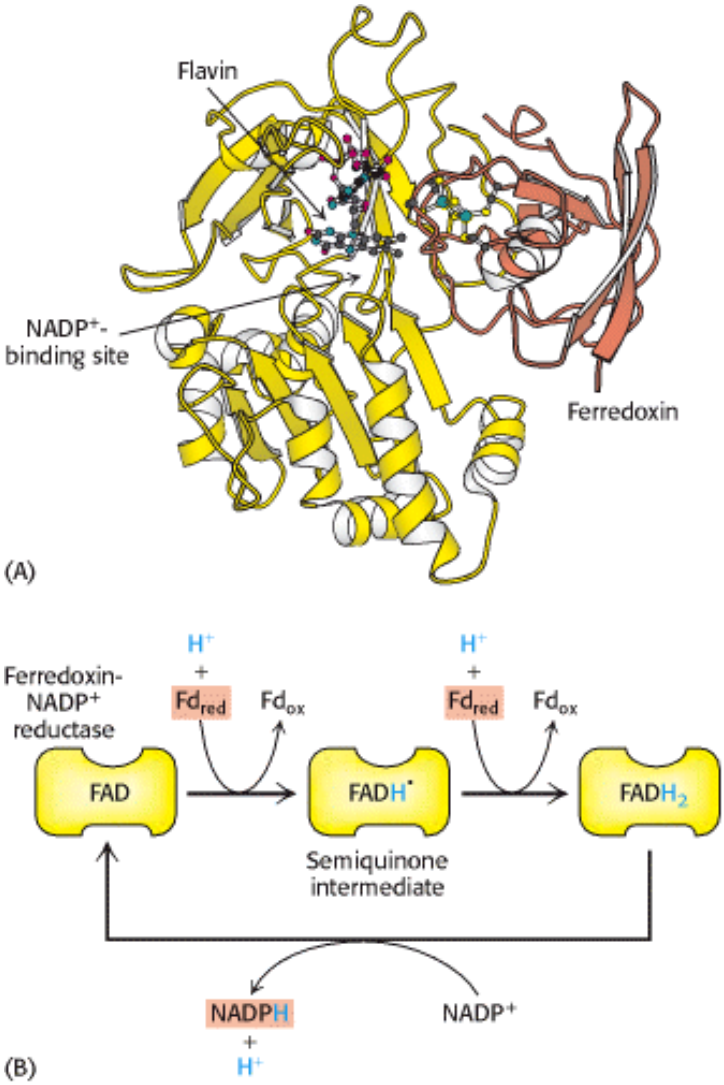


Figure 19.23. Structure and Function of Ferredoxin-NADP$^+$ Reductase. (A) Structure of ferredoxin-NADP$^+$ reductase. This enzyme accepts electrons, one at a time, from ferredoxin (shown in orange). (B) Ferredoxin-NADP$^+$ reductase first accepts one electron from reduced ferredoxin to form a flavin semiquinone intermediate. The enzyme then accepts a second electron to form $FADH_2$, which then transfers two electrons and a proton to NADP$^+$ to form NADPH.

19.4. A Proton Gradient Across the Thylakoid Membrane Drives ATP Synthesis

In 1966, André Jagendorf showed that chloroplasts synthesize ATP in the dark when an artificial pH gradient is imposed across the thylakoid membrane. To create this transient pH gradient, he soaked chloroplasts in a pH 4 buffer for several hours and then rapidly mixed them with a pH 8 buffer containing ADP and P_i. The pH of the stroma suddenly increased to 8, whereas the pH of the thylakoid space remained at 4. *A burst of ATP synthesis then accompanied the disappearance of the pH gradient across the thylakoid membrane* (Figure 19.24). This incisive experiment was one of the first to unequivocally support the hypothesis put forth by Peter Mitchell that ATP synthesis is driven by proton-motive force.

The principles by which ATP synthesis takes place in chloroplasts are nearly identical with those for oxidative phosphorylation. We have seen how light induces electron transfer through photosystems II and I and the cytochrome *bf* complex. At various stages in this process, protons are released into the thylakoid lumen or taken up from the stroma, generating a proton gradient. Such a gradient can be maintained because the thylakoid membrane is essentially

impermeable to protons. *The thylakoid space becomes markedly acidic, with the pH approaching 4. The light-induced transmembrane proton gradient is about 3.5 pH units*. As discussed in Section 18.4, energy inherent in the proton gradient, called the *proton-motive force (Δp)*, is described as the sum of two components: a charge gradient and a chemical gradient. In chloroplasts, nearly all of Δp arises from the pH gradient, whereas, in mitochondria, the contribution from the membrane potential is larger. The reason for this difference is that the thylakoid membrane is quite permeable to Cl^- and Mg^{2+}. The light-induced transfer of H^+ into the thylakoid space is accompanied by the transfer of either Cl^- in the same direction or Mg^{2+} (1 Mg^{2+} per 2 H^+) in the opposite direction. Consequently, electrical neutrality is maintained and no membrane potential is generated. A pH gradient of 3.5 units across the thylakoid membrane corresponds to a proton-motive force of 0.20 V or a ΔG of -4.8 kcal mol^{-1} (-20.0 kJ mol^{-1}).

19.4.1. The ATP Synthase of Chloroplasts Closely Resembles Those of Mitochondria and Prokaryotes

The proton-motive force generated by the light reactions is converted into ATP by the *ATP synthase* of chloroplasts, also called the *CF_1-CF_0 complex* (*C* stands for chloroplast and *F* for factor). CF_1-CF_0 ATP synthase closely resembles the F_1-F_0 complex of mitochondria (Section 18.4.1). CF_0 conducts protons across the thylakoid membrane, whereas CF_1 catalyzes the formation of ATP from ADP and P_i.

CF_0 is embedded in the thylakoid membrane. It consists of four different polypeptide chains known as I (17 kd), II (16.5 kd), III (8 kd), and IV (27 kd) having an estimated stoichiometry of 1:2:12:1. Subunits I, II, and III correspond to subunits **a, b,** and **c,** respectively, of the mitochondrial F_0 subunit, and subunit IV is similar in sequence to subunit **a.** CF_1, the site of ATP synthesis, has a subunit composition $\alpha_3 \beta_3 \gamma \delta \varepsilon$. The β subunits contain the catalytic sites, similar to the F_1 subunit of mitochondrial ATP synthase. Remarkably, β subunits of corn chloroplast ATP synthase are more than 60% identical in amino acid sequence with those of human ATP synthase, despite the passage of approximately 1 billion years since the separation of the plant and animal kingdoms.

Significantly, the membrane orientation of CF_1-CF_0 is reversed compared with that of the mitochondrial ATP synthase (Figure 19.25). Thus, protons flow *out* of the thylakoid lumen through ATP synthase into the stroma. Because CF_1 is on the stromal surface of the thylakoid membrane, the newly synthesized ATP is released directly into the stromal space. Recall that NADPH formed through the action of photosystem I and ferredoxin-$NADP^+$ reductase also is released into the stromal space. Thus, *ATP and NADPH, the products of the light reactions of photosynthesis, are appropriately positioned for the subsequent dark reactions, in which CO_2 is converted into carbohydrate*.

19.4.2. Cyclic Electron Flow Through Photosystem I Leads to the Production of ATP Instead of NADPH

An alternative pathway for electrons arising from P700, the reaction center of photosystem I, contributes to the versatility of photosynthesis. The electron in reduced ferredoxin can be transferred to the cytochrome *bf* complex rather than to $NADP^+$. This electron then flows back through the cytochrome *bf* complex to reduce plastocyanin, which can then be reoxidized by $P700^+$ to complete a cycle. The net outcome of this cyclic flow of electrons is the pumping of protons by the cytochrome *bf* complex. The resulting proton gradient then drives the synthesis of ATP. In this process, called *cyclic photophosphorylation, ATP is generated without the concomitant formation of NADPH* (Figure 19.26). Photosystem II does not participate in cyclic photophosphorylation, and so O_2 is not formed from H_2O. Cyclic photophosphorylation takes place when $NADP^+$ is unavailable to accept electrons from reduced ferredoxin, because of a very high ratio of NADPH to $NADP^+$.

19.4.3. The Absorption of Eight Photons Yields One O_2, Two NADPH, and Three ATP Molecules

We can now estimate the overall stoichiometry for the light reactions. The absorption of 4 photons by photosystem II generates 1 molecule of O_2 and releases 4 protons into the thylakoid lumen. The 2 molecules of plastoquinol are oxidized by the Q cycle of the cytochrome *bf* complex to release 8 protons into the lumen. Finally, the electrons from 4 molecules of reduced plastocyanin are driven to ferredoxin by the absorption of 4 additional photons. The 4 molecules of reduced ferredoxin generate 2 molecules of NADPH. Thus, the overall reaction is:

$$2\,H_2O + 2\,NADP^+ + 10\,H^+_{stroma} \longrightarrow O_2 + 2\,NADPH + 12\,H^+_{lumen}$$

The 12 protons released in the lumen can then flow through ATP synthase. Given the apparent stoichiometry of 12 subunit III components in CF_0, we expect that 12 protons must pass through CF_0 to complete one full rotation of CF_1 and, hence, generate and release 3 molecules of ATP. Given this ratio, the overall reaction is

$$\begin{aligned} 2\,H_2O + 2\,NADP^+ + 10\,H^+_{stroma} &\longrightarrow O_2 + 2\,NADPH + 12\,H^+_{lumen} \\ 3\,ADP^{3-} + 3\,P_i^{2-} + 3\,H^+ + 12\,H^+_{lumen} &\longrightarrow 3\,ATP^{4-} + 3\,H_2O + 12\,H^+_{stroma} \\ \hline 2\,NADP^+ + 3\,ADP^{3-} + 3\,P_i^{2-} + H^+ &\longrightarrow O_2 + 2\,NADPH + 3\,ATP^{4-} + H_2O \end{aligned}$$

Cyclic photophosphorylation is somewhat more productive with regard to ATP synthesis. The absorption of 4 photons by photosystem I results in the release of 8 protons into the lumen by the cytochrome *bf* system. These protons flow through ATP synthase to yield 2 molecules of ATP (assuming the same ratio of ATP molecules generated per proton). Thus, each 2 absorbed photons yield 1 molecule of ATP. No NADPH is produced.

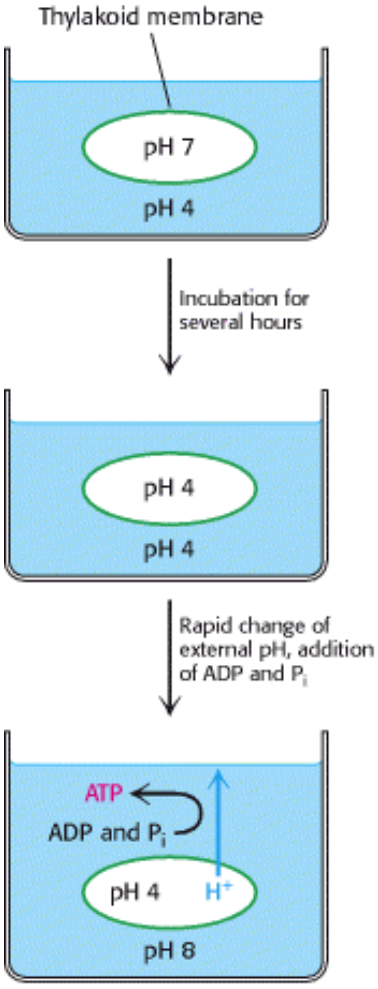


Figure 19.24. Jagendorf's Demonstration. Chloroplasts synthesize ATP after the imposition of a pH gradient.

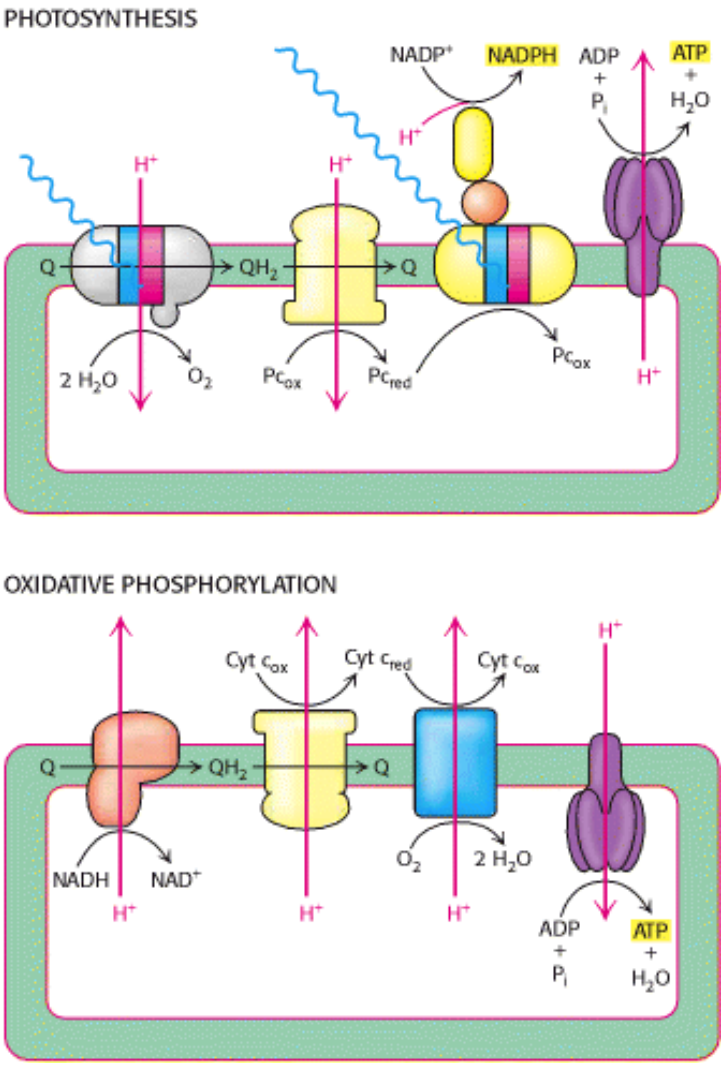


Figure 19.25. Comparison of Photosynthesis and Oxidative Phosphorylation. The light-induced electron transfer in photosynthesis drives protons into the thylakoid lumen. The excess protons flow out of the lumen through ATP synthase to generate ATP in the stroma. In oxidative phosphorylation, electron flow down the electron-transport chain pumps protons out of the mitochondrial matrix. Excess protons from the intermembrane space flow into the matrix through ATP synthase to generate ATP in the matrix.

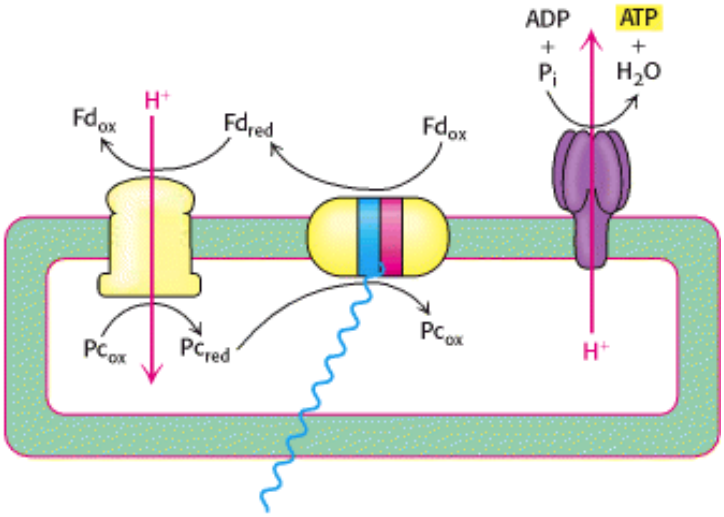


Figure 19.26. Cyclic Photophosphorylation. In this pathway, electrons from reduced ferredoxin are transferred to the cytochrome *bf* complex rather than to ferredoxin-NADP$^+$ reductase. The flow of electrons through cytochrome *bf* pumps

protons into the thylakoid lumen. These protons flow through ATP synthase to generate ATP. Neither NADPH nor O_2 is generated by this pathway.

19.5. Accessory Pigments Funnel Energy Into Reaction Centers

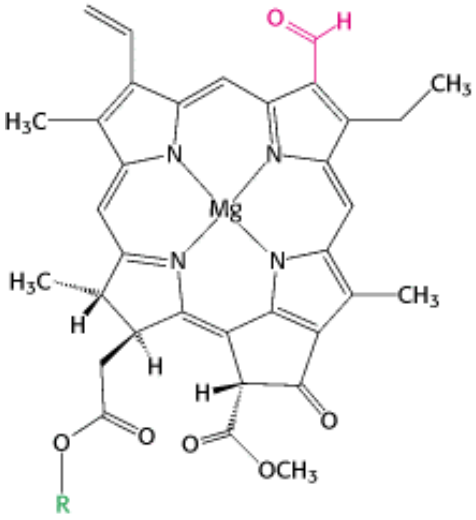
A light-harvesting system that relied only on the chlorophyll *a* molecules of the special pair would be rather inefficient for two reasons. First, chlorophyll *a* molecules absorb light only at specific wavelengths (see Figure 19.6). A large gap is present in the middle of the visible regions between approximately 450 and 650 nm. This gap corresponds to the peak of the solar spectrum, so failure to collect this light would constitute a considerable lost opportunity. Second, even in spectral regions where chlorophyll *a* absorbs light, many photons would pass through without being absorbed, owing to the relatively low density of chlorophyll *a* molecules in a reaction center. Accessory pigments, both additional chlorophylls as well as other classes of molecules, are closely associated with reaction centers. *These pigments absorb light and funnel the energy to the reaction center for conversion into chemical forms*. Indeed, experiments in 1932 by Robert Emerson and William Arnold on *Chlorella* cells (unicellular green algae) demonstrated that only 1 molecule of O_2 was produced for 2500 chlorophyll molecules excited.

19.5.1. Resonance Energy Transfer Allows Energy to Move from the Site of Initial Absorbance to the Reaction Center

How is energy funneled from an associated pigment to a reaction center? We have already seen how the absorption of a photon can lead to electron excitation and transfer (Section 19.2). Another reaction to photon absorption, not leading to electron transfer, is more common. Through electromagnetic interactions through space, the excitation energy can be transferred from one molecule to a nearby molecule (Figure 19.27). The rate of this process, called *resonance energy transfer*, depends strongly on the distance between the energy donor and the energy acceptor molecules; an increase in the distance between the donor and the acceptor by a factor of two typically results in a decrease in the energy-transfer rate by a factor of $2^6 = 64$. For reasons of conservation of energy, energy transfer must be from a donor in the excited state to an acceptor of equal or lower energy. *The excited state of the special pair of chlorophyll molecules is lower in energy than that for single chlorophyll molecules, allowing reaction centers to trap the energy transferred from other molecules* (Figure 19.28).

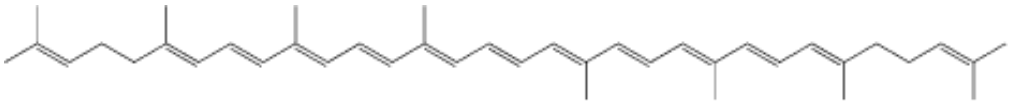
19.5.2. Light-Harvesting Complexes Contain Additional Chlorophylls and Carotinoids

Chlorophyll b and *carotenoids* are important light-harvesting molecules that funnel energy to the reaction center. Chlorophyll *b* differs from chlorophyll *a* in having a formyl group in place of a methyl group. This small difference moves its two major absorption peaks toward the center of the visible region. In particular, chlorophyll *b* efficiently absorbs light with wavelengths between 450 and 500 nm (Figure 19.29).

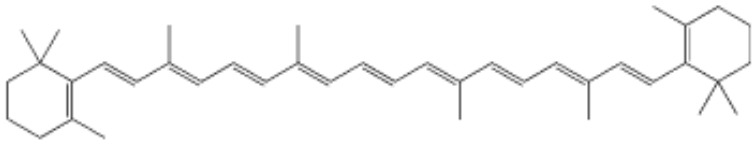


Chlorophyll *b*

Carotenoids are extended polyenes that also absorb light between 400 and 500 nm.



Lycopene



β-Carotene

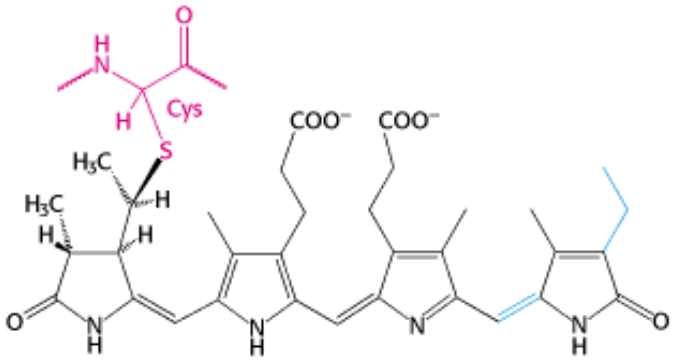
The carotenoids are responsible for most of the yellow and red colors of fruits and flowers, and they provide the brilliance of fall, when the chlorophyll molecules are degraded to reveal the carotenoids.

In addition to their role in transferring energy to reaction centers, the carotenoids serve a safeguarding function. Carotenoids suppress damaging photochemical reactions, particularly those including oxygen, that can be induced by bright sunlight. Indeed, plants lacking carotenoids are quickly killed on exposure to light and oxygen.

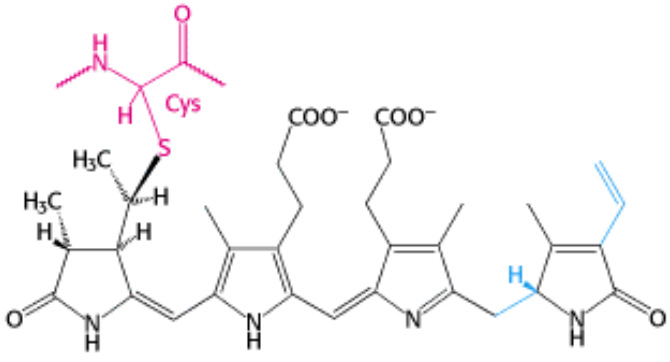
The accessory pigments are arranged in numerous *light-harvesting complexes* that completely surround the reaction center. The 26-kd subunit of light-harvesting complex II (LHC-II) is the most abundant membrane protein in chloroplasts. This subunit binds seven chlorophyll *a* molecules, six chlorophyll *b* molecules, and two carotenoid molecules. Similar lightharvesting assemblies exist in photosynthetic bacteria (Figure 19.30).

19.5.3. Phycobilisomes Serve as Molecular Light Pipes in Cyanobacteria and Red Algae

Little blue or red light reaches algae living at a depth of a meter or more in seawater, because such light is absorbed by water and by chlorophyll molecules in organisms lying above. How can photosynthetic organisms survive under such conditions? Cyanobacteria (blue-green algae) and red algae contain large protein assemblies called *phycobilisomes* that enable them to harvest the green and yellow light that penetrates to their ecological niche. Phycobilisomes are bound to the outer face of the thylakoid membrane, where they serve as light-absorbing antennas to funnel excitation energy into the reaction centers of photosystem II. They absorb maximally in the 470- to 650-nm region, in the valley between the blue and far-red absorption peaks of chlorophyll *a*. Phycobilisomes are very large assemblies (several million daltons) of many *phycobiliprotein* subunits, each containing many covalently attached *bilin* prosthetic groups, as well as linker polypeptides (Figure 19.31). Phycobilisomes contain hundreds of bilins. Phycocyanobilin and phycoerythrobilin are the two most common ones.



Peptide-linked phycocyanobilin



Peptide-linked phycoerythrobilin

The geometrical arrangement of phycobiliproteins in phycobilisomes (Figure 19.32), as well as their spectral properties, contributes to the efficiency of energy transfer, which is greater than 95%. Excitation energy absorbed by phycoerythrin subunits at the periphery of these antennas appears at the reaction center in less than 100 ps. Phycobilisomes are elegantly designed light pipes that enable algae to occupy ecological niches that would not support organisms relying solely on chlorophyll for the trapping of light.

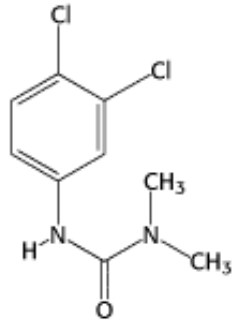
19.5.4. Components of Photosynthesis Are Highly Organized

The complexity of photosynthesis, seen already in the elaborate interplay of complex components, extends even to the placement of the components in the thylakoid membranes. *Thylakoid membranes of most plants are differentiated into stacked* (appressed) and *unstacked* (nonappressed) regions (see Figures 19.1 and 19.3). Stacking increases the amount of thylakoid membrane in a given chloroplast volume. Both regions surround a common internal thylakoid space, but only unstacked regions make direct contact with the chloroplast stroma. Stacked and unstacked regions differ in the nature of their photosynthetic assemblies (Figure 19.33). Photosystem I and ATP synthase are located almost exclusively in unstacked regions, whereas photosystem II is present mostly in stacked regions. The cytochrome *bf* complex is found in both regions. Indeed, this complex rapidly moves back and forth between the stacked and unstacked regions. Plastoquinone and plastocyanin are the mobile carriers of electrons between assemblies located in different regions of the thylakoid membrane. A common internal thylakoid space enables protons liberated by photosystem II in stacked membranes to be utilized by ATP synthase molecules that are located far away in unstacked membranes.

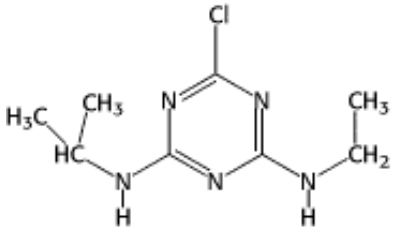
What is the functional significance of this lateral differentiation of the thylakoid membrane system? The positioning of photosystem I in the unstacked membranes also gives it direct access to the stroma for the reduction of $NADP^+$. ATP synthase, too, is located in the unstacked region to provide space for its large CF_1 globule and to give access to ADP. In contrast, the tight quarters of the appressed region pose no problem for photosystem II, which interacts with a small polar electron donor (H_2O) and a highly lipid soluble electron carrier (plastoquinone).

19.5.5. Many Herbicides Inhibit the Light Reactions of Photosynthesis

Many commercial herbicides kill weeds by interfering with the action of photosystem II or photosystem I. Inhibitors of photosystem II block electron flow, whereas inhibitors of photosystem I divert electrons from the terminal part of this photosystem. Photosystem II inhibitors include urea derivatives such as *diuron* and triazine derivatives such as *atrazine*. These chemicals bind to the Q_B site of the D1 subunit of photosystem II and block the formation of plastoquinol (QH_2).



Diuron



Atrazine

Paraquat (1,1′-dimethyl-4-4′-bipyridinium) is an inhibitor of photosystem I. Paraquat, a dication, can accept electrons from photosystem I to become a radical. This radical reacts with O_2 to produce reactive oxygen species such as superoxide and hydroxyl radical (OH·). Such reactive oxygen species react with double bonds in membrane lipids, damaging the membrane (Section 18.3.6).

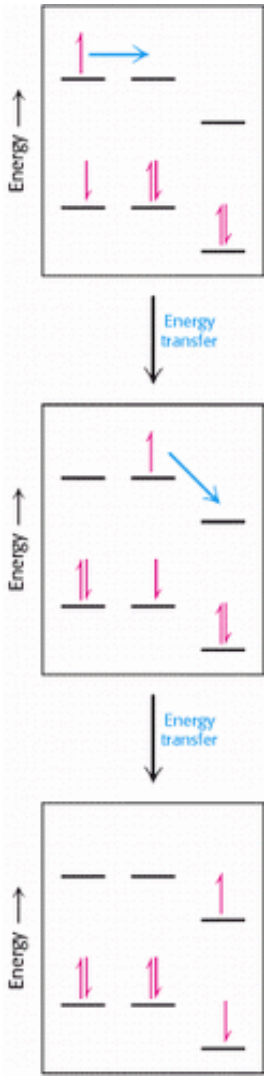


Figure 19.27. Resonance Energy Transfer. Energy, absorbed by one molecule, can be transferred to nearby molecules with excited states of equal or lower energy.

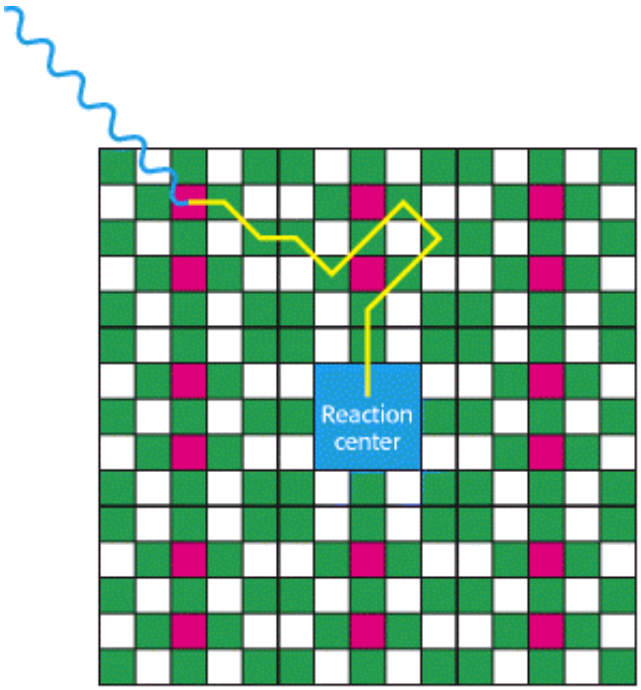


Figure 19.28. Energy Transfer from Accessory Pigments to Reaction Centers. Light energy absorbed by accessory chlorophyll molecules or other pigments can be transferred to reaction centers, where it drives photoinduced charge separation. The green squares represent accessory chlorophyll molecules and the red squares carotenoid molecules; the white squares designate protein.

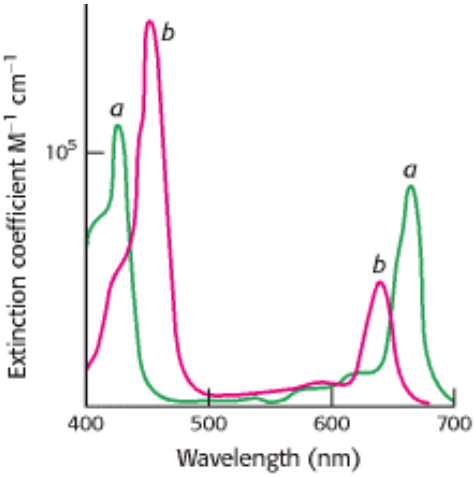


Figure 19.29. Absorption Spectra of Chlorophyll. *A* and *B* .

Figure 19.30. Structure of a Light-Harvesting Complex. Eight polypeptides, each of which binds three chlorophyll molecules (green) and a carotenoid molecule (red), surround a central cavity that contains the reaction center.

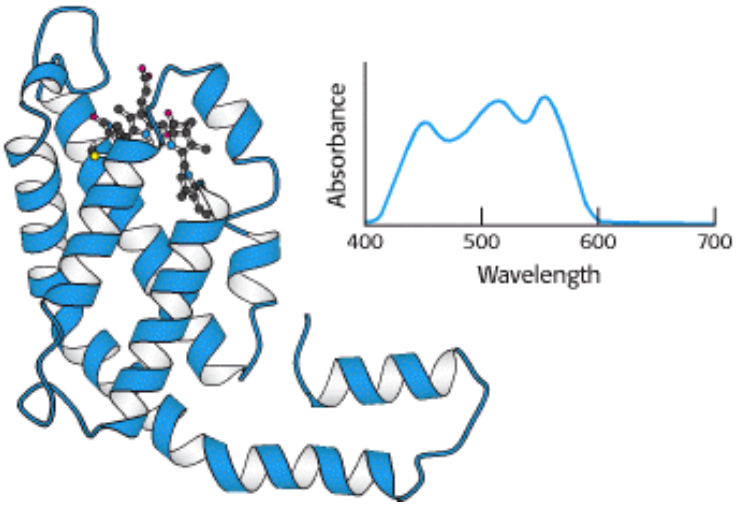


Figure 19.31. Structure of a Phycobilisome Subunit. This protein, a phycoerythrin, contains a phycoerythrobilin linked to a cysteine residue. The inset shows the absorption spectrum of a phycoerythrin.

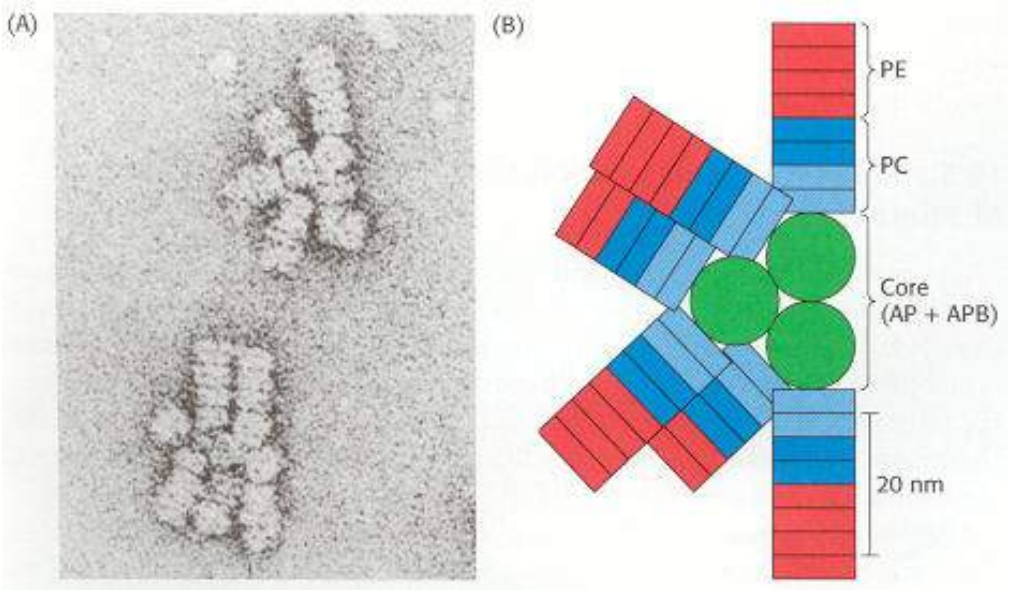


Figure 19.32. Structure of a Phycobilisome. (A) Electron micrograph of phycobilisomes from a cyanobacterium (*Synechocystis*). (B) Schematic representation of a phycobilisome from the cyanobacterium *Synechocystis* 6701. Rods containing phycoerythrin (PE) and phycocyanin (PC) emerge from a core made of allophycocyanin (AP) and allophycocyanin B (APB). The core region binds to the thylakoid membrane. [(A) Courtesy of Dr. Robley Williams and Dr. Alexander Glazer; (B) after a drawing kindly provided by Dr. Alexander Glazer.]

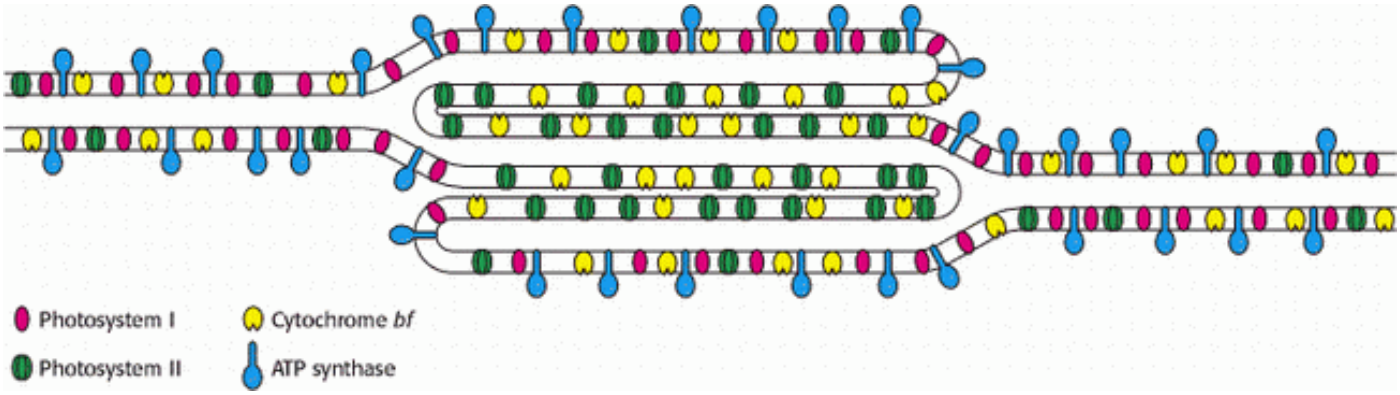


Figure 19.33. Location of Photosynthesis Components. Photosynthetic assemblies are differentially distributed in the stacked (appressed) and unstacked (nonapressed) regions of thylakoid membranes. [After a drawing kindly provided by Dr. Jan M. Anderson and Dr. Bertil Andersson.]

19.6. The Ability to Convert Light Into Chemical Energy Is Ancient

The ability to convert light energy into chemical energy is a significant evolutionary advantage. Indeed, photosynthesis arose early in the history of life on Earth, which began about 3.5 billion years ago. Geolog- ical evidence suggests that oxygenic photosynthesis became important approximately 2 billion years ago. Anoxygenic photosynthetic systems existed even earlier (Table 19.1). The photosynthetic system of the nonsulfur purple bacterium *Rhodospeudomonas viridis* has most features common to oxygenic photosynthetic systems and clearly predates them. Green sulfur bacteria such as *Chlorobium thiosulfatophilum* carry out a reaction that also seems to have appeared before oxygenic photosynthesis and is even more similar to oxygenic photosynthesis than that of *R. viridis* . Reduced sulfur species such as H_2S are electron donors in the overall photosynthetic reaction:

$$CO_2 + 2\,H_2S \xrightarrow{\text{Light}} (CH_2O) + 2\,S + H_2O$$

Nonetheless, photosynthesis did not evolve immediately at the origin of life. The failure to discover photosynthesis in the domain of Archaea implies that photosynthesis evolved exclusively in the domain of Bacteria. Eukaryotes appropriated through endosymbiosis the basic photosynthetic units that were the products of bacterial evolution. All domains of life do have electron-transport chains in common, however. As we have seen, components such as the ubiquinone-cytochrome *c* oxidoreductase and cytochrome *bf* family are present in both respiratory and photosynthetic electron-transport chains. These components were the foundations on which light-energy-capturing systems evolved.

Table 19.1. Major Groups of Photosynthetic Prokaryotes

Bacteria	Photosynthetic electron donor	O_2 use
Green sulfur	H_2, H_2S, S	Anoxygenic
Green nonsulfur	Variety of amino acids and organic acids	Anoxygenic
Purple sulfur	H_2, H_2S, S	Anoxygenic
Purple nonsulfur	Usually organic molecules	Anoxygenic

Cyanobacteria	H_2O	Oxygenic

Summary

Photosynthesis Takes Place in Chloroplasts

The proteins that participate in the light reactions of photosynthesis are located in the thylakoid membranes of chloroplasts. The light reactions result in (1) the creation of reducing power for the production of NADPH, (2) the generation of a transmembrane proton gradient for the formation of ATP, and (3) the production of O_2.

Light Absorption by Chlorophyll Induces Electron Transfer

Chlorophyll molecules—tetrapyrroles with a central magnesium ion—absorb light quite efficiently because they are polyenes. An electron excited to a high-energy state by the absorption of a photon can move to nearby electron acceptors. In photosynthesis, an excited electron leaves a pair of associated chlorophyll molecules known as the special pair. The functional core of photosynthesis, a reaction center, from a photosynthetic bacterium has been studied in great detail. In this system, the electron moves from the special pair (containing bacteriochlorophyll) to a bacteriopheophytin (a bacteriochlorophyll lacking the central magnesium ion) to quinones. The reduction of quinones leads to the generation of a proton gradient, which drives ATP synthesis in a manner analogous to that of oxidative phosphorylation.

Two Photosystems Generate a Proton Gradient and NADPH in Oxygenic Photosynthesis.

Photosynthesis in green plants is mediated by two linked photosystems. In photosystem II, excitation of P680, a special pair of chlorophyll molecules located at the interface of two similar subunits, leads to electron transfer to plastoquinone in a manner analogous to that for the bacte-rial reaction center. The electrons are replenished by the extraction of electrons from water at a center containing four manganese ions. One molecule of O_2 is generated at this center for each four electrons transferred. The plastoquinol produced at photosystem II is reoxidized by the cytochrome *bf* complex, which transfers the electrons to plastocyanin, a soluble copper protein. From plastocyanin, the electrons enter photosystem I. In photosytem I, excitation of the special pair P700 releases electrons that flow to ferredoxin, a powerful reductant. Ferredoxin-$NADP^+$ reductase, a flavoprotein located on the stromal side of the membrane, then catalyzes the formation of NADPH. A proton gradient is generated as electrons pass through photosystem II, through the cytochrome *bf* complex, and through ferredoxin-$NADP^+$ reductase.

A Proton Gradient Across the Thylakoid Membrane Drives ATP Synthesis

The proton gradient across the thylakoid membrane creates a proton-motive force, used by ATP synthase to form ATP. The ATP synthase of chloroplasts (also called CF_0-CF_1) closely resembles the ATPsynthesizing assemblies of bacteria and mitochondria (F_0-F_1). If the NADPH:$NADP^+$ ratio is high, electrons transferred to ferredoxin by photosystem I can reenter the cytochrome *bf* complex. This process, called cyclic photophosphorylation, leads to the generation of a proton gradient by the cytochrome *bf* complex without the formation of NADPH or O_2.

Accessory Pigments Funnel Energy into Reaction Centers

Light-harvesting complexes that surround the reaction centers contain additional molecules of chlorophyll *a*, as well as carotenoids and chlorophyll *b* molecules, which absorb light in the center of the visible spectrum. These accessory pigments increase efficiency in light capture by absorbing light and transferring the energy to reaction centers through

resonance energy transfer. In blue-green and red algae, phycobilisomes—large protein assemblies with bound pigments called bilins—act as light-absorbing antennas.

The Ability to Convert Light into Chemical Energy Is Ancient

The photosystems have structural features in common that suggest a common evolutionary origin. Similarities in organization and molecular structure to those of oxidative phosphorylation suggest that the photosynthetic apparatus evolved from an early energy-transduction system.

Key Terms

light reaction

chloroplast

stroma

thylakoid

granum

chlorophyll *a*

photoinduced charge separation

reaction center

photosystem I (PS I)

photosystem II (PS II)

special pair

P960

P680

manganese center

cytochrome *bf*

P700

Z scheme of photosynthesis

proton-motive force (Δp)

ATP synthase (CF_1-CF_0 complex)

cyclic photophosphorylation

carotenoid

light-harvesting complex

phycobilisome

Problems

1. *Electron transfer*. Calculate the $\Delta E'_0$ and $\Delta G^{\circ\prime}$ for the reduction of $NADP^+$ by ferredoxin. Use data given in Table 18.1 in Section 18.2.1.

   See answer

2. *To boldly go.* (a) It can be argued that, if life were to exist elsewhere in the universe, it would require some process like photosynthesis. Why is this a reasonable argument? (b) If the Enterprise were to land on a distant plant and find no measurable oxygen in the atmosphere, could the crew conclude that photosynthesis is not taking place?

   See answer

3. *Weed killer 1*. Dichlorophenyldimethylurea (DCMU), a herbicide, interferes with photophosphorylation and O_2 evolution. However, it does not block O_2 evolution in the presence of an artificial electron acceptor such as ferricyanide. Propose a site for the inhibitory action of DCMU.

   See answer

4. *Weed killer 2*. Predict the effect of the herbicide dichlorophenyldimethylurea (DCMU) on a plant's ability to perform cyclic photophosphorylation.

   See answer

5. *Infrared harvest.* Consider the relation between the energy of a photon and its wavelength.

   (a) Some bacteria are able to harvest 1000-nm light. What is the energy (in kilocalories or kilojoules) of a mole (also called an einstein) of 1000-nm photons?

   (b) What is the maximum increase in redox potential that can be induced by a 1000-nm photon?

   (c) What is the minimum number of 1000-nm photons needed to form ATP from ADP and P_i? Assume a ΔG of 12 kcal mol^{-1} (50 kJ mol^{-1}) for the phosphorylation reaction.

   See answer

6. *Missing acceptors.* Suppose that a bacterial reaction center containing only the special pair and the quinones was prepared. Given the separation of 22 Å between the special pair and the closest quinone, estimate the rate of electron transfer between the excited special pair and this quinone.

   See answer

7. *Self-preservation.* Blue-green bacteria deprived of a source of nitrogen digest their least-essential proteins. Which phycobilisome component is likely to be degraded first under starvation conditions?

See answer

8. *Close approach.* Suppose that energy transfer between two chlorophyll *a* molecules separated by 10 Å occurs in 10 picoseconds. Suppose that this distance is increased to 20 Å with all other factors remaining the same. How long would energy transfer take?

See answer

Mechanism Problem

9. *Hill reaction.* In 1939, Robert Hill discovered that chloroplasts evolve O_2 when they are illuminated in the presence of an artificial electron acceptor such as ferricyanide $[Fe^{3+}(CN)_6]^{3-}$. Ferricyanide is reduced to ferrocyanide $[Fe^{2+}(CN)_6]^{4-}$ in this process. No NADPH or reduced plastocyanin is produced. Propose a mechanism for the Hill reaction.

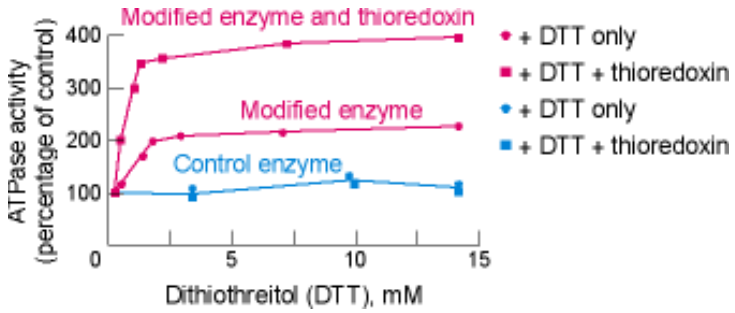
See answer

Data Interpretation and Chapter Integration Problem

10. *The same, but different.* The $\alpha_3\beta_3\gamma$ complex of mitochondrial or chloroplast ATP synthase will function as an ATPase in vitro. The chloroplast enzyme (both synthase and ATPase activity) is sensitive to redox control, whereas the mitochondrial enzyme is not. To determine where the enzymes differ, a segment of the mitochondrial γ subunit was removed and replaced with the equivalent segment from the chloroplast γ subunit. The ATPase activity of the modified enzyme was then measured as a function of redox conditions.

(a) What is the redox regulator of the ATP synthase in vivo?

The graph below shows the ATPase activity of modified and control enzymes under various redox conditions.



[Data after O. Bald, et al., 2000. *J Biol. Chem.*, 275: 12757–12762.]

(b) What is the effect of increasing the reducing power of the reaction mixture for the control and modified enzymes?

(c) What was the effect of the addition of thioredoxin? How did these results differ from those in the presence of DTT alone? Suggest a possible explanation for the difference.

(d) Did the researchers succeed in identifying the region of the γ subunit responsible for redox regulation?

(e) What is the biological rationale of regulation by high concentrations of reducing agents?

(f) What amino acids in the γ subunit are most likely affected by the reducing conditions?

(g) What experiments might confirm your answer to part *e*?

See answer

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20. The Calvin Cycle and the Pentose Phosphate Pathway

Photosynthesis proceeds in two parts: the light reactions and the dark reactions. The light reactions, which were discussed in Chapter 19, transform light energy into ATP and biosynthetic reducing power, NADPH. The dark reactions, which constitute the Calvin cycle, named after Melvin Calvin, the biochemist who elucidated the pathway, reduce carbon atoms from their fully oxidized state as carbon dioxide to the more reduced state as a hexose. The components of the Calvin cycle and called the dark reactions because, in contrast with the light reactions, these reactions do not directly depend on the presence of light.

In addition to ATP, the dark reactions require reducing power in the form of *NADPH, the currency of readily available reducing power in cells*. The phosphoryl group on the 2′-hydroxyl group of one of the ribose units of NADPH distinguishes NADPH from NADH. There is a *fundamental distinction between NADPH and NADH in biochemistry: NADH is oxidized by the respiratory chain to generate ATP, whereas NADPH serves as a reductant in biosynthetic processes*.

The second half of this chapter examines a pathway common to all organisms, known variously as the pentose phosphate pathway, the hexose monophosphate pathway, the phosphogluconate pathway, or the pentose shunt. The pathway provides a means by which glucose can be oxidized to generate NADPH and is the source of much of the NADPH that is needed for the biosynthesis of many biomolecules, most notably fats. We will observe the use of NADPH in many of the biosynthetic reactions considered in Part III of this text. The pentose phosphate pathway can also be used for the catabolism of pentose sugars from the diet, the synthesis of pentose sugars for nucleotide biosynthesis, and the catabolism and synthesis of less common four- and seven-carbon sugars. The pentose phosphate pathway and the Calvin cycle have in common several enzymes and intermediates that attest to an evolutionary kinship. Like glycolysis and gluconeogenesis (Chapter 16), these pathways are mirror images of one another: the Calvin cycle uses NADPH to reduce carbon dioxide to generate hexoses, whereas the pentose phosphate pathway breaks down glucose into carbon dioxide to generate NADPH. The Calvin cycle is sometimes referred to as the *reductive pentose phosphate pathway*.

Atmospheric carbon dioxide measurements as Mauna Loa, Hawaii. These measurements show annual cycles resulting from seasonal variation in carbon dioxide fixation by the Calvin cycle in terrestrial plants. Much of this fixation takes place in rain forests, which account for approximately 50% of terrestrial fixation. [Dennis Potokar/Photo Researchers.]

20.1. The Calvin Cycle Synthesizes Hexoses from Carbon Dioxide and Water

We saw in Chapter 16 that glucose could be formed from noncarbohydrate precursors, such as lactate and amino acids, by gluconeogenesis. The synthesis of glucose from these compounds is simplified because the carbons are already incorporated into relatively complex organic molecules. In contrast, the source of the carbon atoms in the Calvin cycle is the simple molecule carbon dioxide. In this extremely important process, *carbon dioxide gas is trapped in a form that is useful for many processes*. The Calvin cycle brings into living systems the carbon atoms that will become constituents of nucleic acids, proteins, and fats. Photosynthetic organisms are called *autotrophs* (literally "self-feeders") because they can synthesize glucose from carbon dioxide and water, by using sunlight as an energy source, and then recover some of this energy from the synthesized glucose through the glycolytic pathway and aerobic metabolism. Organisms that obtain energy from chemical fuels only are called *heterotrophs*, which ultimately depend on autotrophs for their fuel. The Calvin cycle also differs from gluconeogenesis in where it takes place in photosynthetic eukaryotes. Whereas gluconeogenesis takes place in the cytoplasm, the Calvin cycle takes place in the stroma of chloroplasts, the photosynthetic organelles.

The Calvin cycle comprises three stages (Figure 20.1):

1. The fixation of CO_2 by ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate.

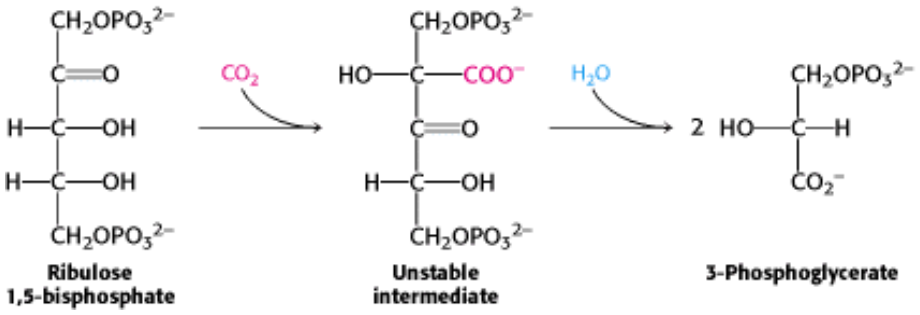
2. The reduction of 3-phosphoglycerate to form hexose sugars.

3. The regeneration of ribulose 1,5-bisphosphate so that more CO_2 can be fixed.

Although we will focus on the Calvin cycle, other means of fixing carbon dioxide into hexose sugars exist in the photosynthetic world, notably a version of the citric acid cycle running in reverse.

20.1.1. Carbon Dioxide Reacts with Ribulose 1,5-bisphosphate to Form Two Molecules of 3-Phosphoglycerate

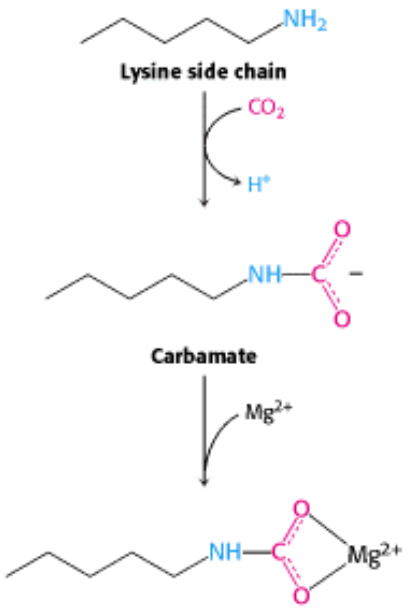
The first step in the Calvin cycle is the fixation of CO_2. The CO_2 molecule condenses with ribulose 1,5-bisphosphate to form an unstable six-carbon compound, which is rapidly hydrolyzed to two molecules of 3-phosphoglycerate.



The initial incorporation of CO_2 into 3-phosphoglycerate was revealed through the use of a carbon-14 radioactive tracer (Figure 20.2). This highly exergonic reaction [$\Delta G^{\circ\prime} = -12.4$ kcal mol^{-1} (-51.9 kJ mol^{-1})] is catalyzed by *ribulose 1,5-bisphosphate carboxylase/oxygenase* (usually called *rubisco*), an enzyme located on the stromal surface of the thylakoid membranes of chloroplasts. This important reaction is the rate-limiting step in hexose synthesis. Rubisco in chloroplasts consists of eight large (L, 55-kd) subunits and eight small (S, 13-kd) ones (Figure 20.3). Each L chain contains a catalytic site and a regulatory site. The S chains enhance the catalytic activity of the L chains. This enzyme is very abundant in chloroplasts, constituting more than 16% of their total protein. In fact, rubisco is the most abundant enzyme

and probably the most abundant protein in the biosphere. Large amounts are present because rubisco is a slow enzyme; its maximal catalytic rate is only 3 s^{-1}.

Rubisco requires a bound divalent metal ion for activity, usually magnesium ion. Like the zinc ion in the active site of carbonic anhydrase (Section 9.2.1), this metal ion serves to activate a bound substrate molecule by stabilizing a negative charge. Interestingly, a CO_2 molecule other than the substrate is required to complete the assembly of the Mg^{2+} binding site in rubisco. This CO_2 molecule adds to the uncharged ε-amino group of lysine 201 to form a *carbamate*. This negatively charged adduct then binds the Mg^{2+} ion. The formation of the carbamate is facilitated by the enzyme *rubisco activase*, although it will also form spontaneously at a lower rate.



The metal center plays a key role in binding ribulose 1,5-bisphosphate and activating it so that it will react with CO_2 (Figure 20.4). Ribulose 1,5-bisphosphate binds to Mg^{2+} through its keto group and an adjacent hydroxyl group. This complex is readily deprotonated to form an enediolate intermediate. This reactive species, analogous to the zinc-hydroxide species in carbonic anhydrase (Section 9.2.2), couples with CO_2, forming the new carbon-carbon bond. The resulting product is coordinated to the Mg^{2+} ion through three groups, including the newly formed carboxylate. A molecule of H_2O is then added to this β-ketoacid to form an intermediate that cleaves to form two molecules of 3-phosphoglycerate (Figure 20.5).

20.1.2. Catalytic Imperfection: Rubisco Also Catalyzes a Wasteful Oxygenase Reaction

The reactive intermediate generated on the Mg^{2+} ion sometimes reacts with O_2 instead of CO_2. Thus, rubisco also catalyzes a deleterious oxygenase reaction. The products of this reaction are *phosphoglycolate* and *3-phosphoglycerate* (Figure 20.6). The rate of the carboxylase reaction is four times that of the oxygenase reaction under normal atmospheric conditions at 25°C; the stromal concentration of CO_2 is then 10 μM and that of O_2 is 250 μM. The oxygenase reaction, like the carboxylase reaction, requires that lysine 201 be in the carbamate form. Because this carbamate forms only in the presence of CO_2, this property would prevent rubisco from catalyzing the oxygenase reaction exclusively when CO_2 is absent.

Phosphoglycolate is not a versatile metabolite. A salvage pathway recovers part of its carbon skeleton (Figure 20.7). A specific phosphatase converts phosphoglycolate into *glycolate*, which enters *peroxisomes* (also called *microbodies*; Figure 20.8). Glycolate is then oxidized to *glyoxylate* by glycolate oxidase, an enzyme with a flavin mononucleotide prosthetic group. The H_2O_2 produced in this reaction is cleaved by catalase to H_2O and O_2. Transamination of glyoxylate then yields *glycine*. Two glycine molecules can be used to form serine, a potential precursor of glucose, with

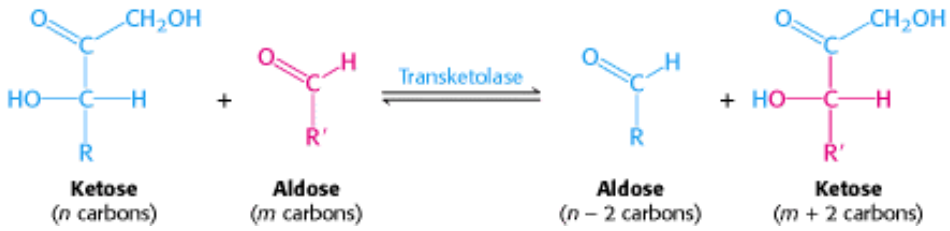
the release of CO_2 and ammonia (NH_4^+). The ammonia, used in the synthesis of nitrogen-containing compounds, is salvaged by glutamine synthetase reaction.

This salvage pathway serves to recycle three of the four carbon atoms of two molecules of glycolate. However, one carbon atom is lost as CO_2. This process is called *photorespiration* because O_2 is consumed and CO_2 is released. Photorespiration is wasteful because organic carbon is converted into CO_2 without the production of ATP, NADPH, or another energy-rich metabolite. Moreover, the oxygenase activity increases more rapidly with temperature than the carboxylase activity, presenting a problem for trop-ical plants (Section 20.2.3). Evolutionary processes have presumably enhanced the preference of rubisco for carboxylation. For instance, the rubisco of higher plants is eightfold as specific for carboxylation as that of photosynthetic bacteria.

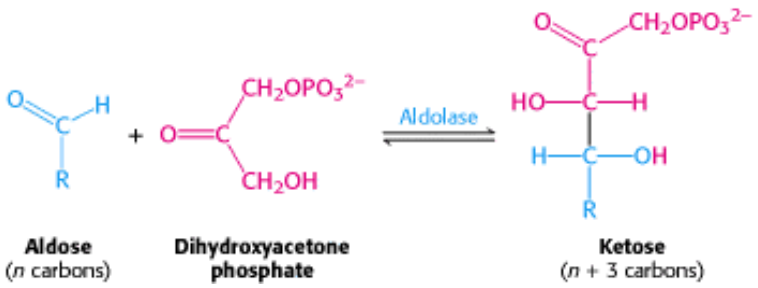
20.1.3. Hexose Phosphates Are Made from Phosphoglycerate, and Ribulose 1,5-bisphosphate Is Regenerated

The 3-phosphoglycerate product of rubisco is next converted into three forms of hexose phosphate: glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phosphate. Recall that these isomers are readily interconvertible (Sections 16.1.2 and 16.1.11). The steps in this conversion (Figure 20.9) are like those of the gluconeogenic pathway (Section 16.3.1), except that glyceraldehyde 3-phosphate dehydrogenase in chloroplasts, which generates glyceraldehyde 3-phosphate (GAP), is specific for NADPH rather than NADH. Alternatively, the glyceraldehyde 3-phosphate can be transported to the cytosol for glucose synthesis. These reactions and that catalyzed by rubisco bring CO_2 to the level of a hexose, converting CO_2 into a chemical fuel at the expense of NADPH and ATP generated from the light reactions.

The third phase of the Calvin cycle is the regeneration of ribulose 1,5-bisphosphate, the acceptor of CO_2 in the first step. The problem is to construct a five-carbon sugar from six-carbon and three-carbon sugars. A transketolase and an aldolase play the major role in the rearrangement of the carbon atoms. The *transketolase*, which we will see again in the pentose phosphate pathway (Section 20.2.3), requires the coenzyme thiamine pyrophosphate (TPP) to transfer a two-carbon unit ($CO\text{-}CH_2OH$) from a ketose to an aldose.



We will consider the mechanism of transketolase when we meet it again in the pentose phosphate pathway (Section 20.3.2). *Aldolase*, which we have already encountered in glycolysis (Section 16.1.3), catalyzes an aldol condensation between dihydroxyacetone phosphate and an aldehyde. This enzyme is highly specific for dihydroxyacetone phosphate, but it accepts a wide variety of aldehydes.



With these enzymes, the construction of the five-carbon sugar proceeds as shown in Figure 20.10.

Finally, ribose-5-phosphate is converted into ribulose 5-phosphate by *phosphopentose isomerase* while xylulose 5-phosphate is converted into ribulose 5-phosphate by *phosphopentose epimerase*. Ribulose 5-phosphate is converted into ribulose 1,5-bisphosphate through the action of *phosphoribulose kinase* (Figure 20.11). The sum of these reactions is

$$\begin{aligned}\text{Fructose 6-phosphate} + 2\ \text{glyceraldehyde 3-phosphate} \\ + \text{dihydroxyacetone phosphate} + 3\ \text{ATP} \longrightarrow \\ 3\ \text{ribulose 1,5-bisphosphate} + 3\ \text{ADP}\end{aligned}$$

This series of reactions completes the Calvin cycle (Figure 20.12). The sum of all the reactions results in the generation of a hexose and the regeneration of the starting compound, ribulose 5-phosphate. In essence, ribulose 1,5-bisphosphate acts catalytically, similarly to oxaloacetate in the citric acid cycle.

20.1.4. Three Molecules of ATP and Two Molecules of NADPH Are Used to Bring Carbon Dioxide to the Level of a Hexose

What is the energy expenditure for synthesizing a hexose? Six rounds of the Calvin cycle are required, because one carbon atom is reduced in each round. Twelve molecules of ATP are expended in phosphorylating 12 molecules of 3-phosphoglycerate to 1,3-bisphosphoglycerate, and 12 molecules of NADPH are consumed in reducing 12 molecules of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate. An additional six molecules of ATP are spent in regenerating ribulose 1,5-bisphosphate. We can now write a balanced equation for the net reaction of the Calvin cycle.

$$\begin{aligned}6\,CO_2 + 18\,ATP + 12\,NADPH + 12\,H_2O \longrightarrow \\ C_6H_{12}O_6 + 18\,ADP + 18\,P_i + 12\,NADP^+ + 6\,H^+\end{aligned}$$

Thus, three molecules of ATP and two molecules of NADPH are consumed in incorporating a single CO_2 molecule into a hexose such as glucose or fructose.

20.1.5. Starch and Sucrose Are the Major Carbohydrate Stores in Plants

Plants contain two major storage forms of sugar: *starch* and *sucrose*. Starch, like its animal counterpart glycogen, is a polymer of glucose residues, but it is less branched than glycogen because it contains a smaller proportion of α-1,6-glycosidic linkages (Section 11.2.2). Another difference is that ADP-glucose, not UDP-glucose, is the activated precursor. Starch is synthesized and stored in chloroplasts.

In contrast, sucrose (common table sugar), a disaccharide, is synthesized in the cytosol. Plants lack the ability to transport hexose phosphates across the chloroplast membrane, but an abundant phosphate translocator mediates the transport of triose phosphates from chloroplasts to the cytosol in exchange for phosphate. Fructose 6-phosphate formed from triose phosphates joins the glucose unit of UDP-glucose to form sucrose 6-phosphate (Figure 20.13). Hydrolysis of the phosphate ester yields sucrose, a readily transportable and mobilizable sugar that is stored in many plant cells, as in sugar beets and sugar cane.

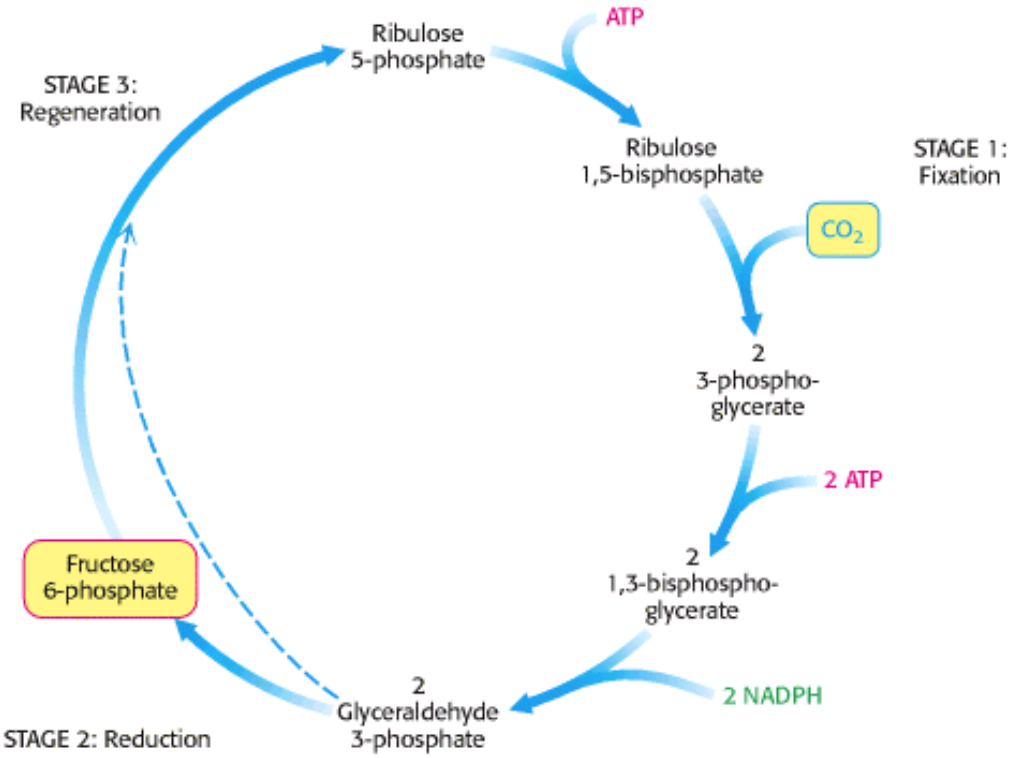


Figure 20.1. Calvin Cycle. The Calvin cycle consists of three stages. Stage 1 is the fixation of carbon by the carboxylation of ribulose 1,5-bisphosphate. Stage 2 is the reduction of the fixed carbon to begin the synthesis of hexose. Stage 3 is the regeneration of the starting compound, ribulose 1,5-bisphosphate.

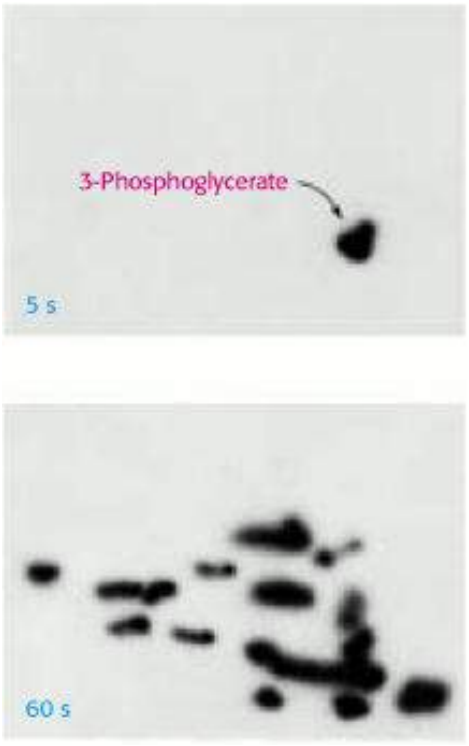


Figure 20.2. Tracing the Fate of Carbon Dioxide. Radioactivity from $^{14}CO_2$ is incorporated into 3-phosphoglycerate within 5 s in irradiated cultures of algae. After 60 s, the radioactivity appears in many compounds, the intermediates within the Calvin cycle. [Courtesy of Dr. J. A. Bassham.]

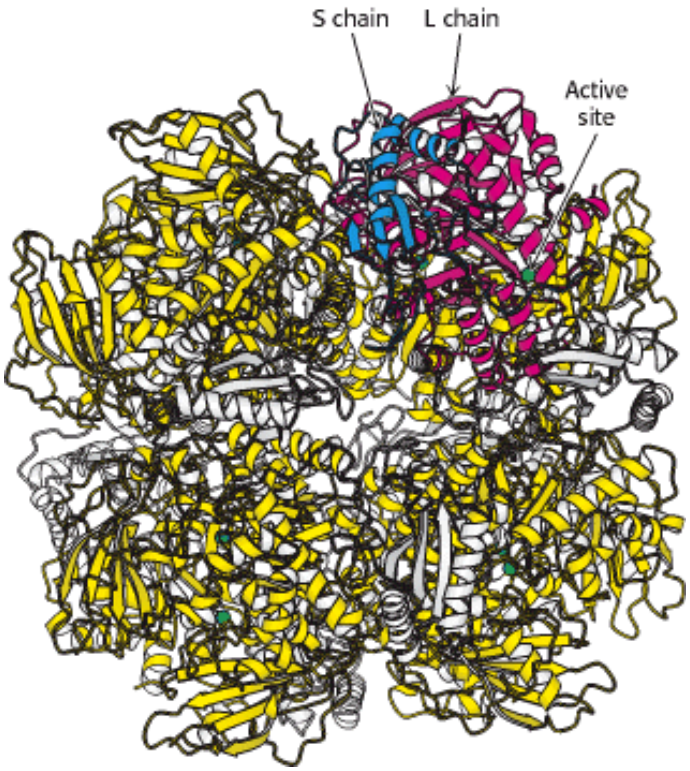


Figure 20.3. Structure of Rubisco. The enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) comprises eight large subunits (one shown in red and the others in yellow) and eight small subunits (one shown in blue and the others in white). The active sites lie in the large subunits.

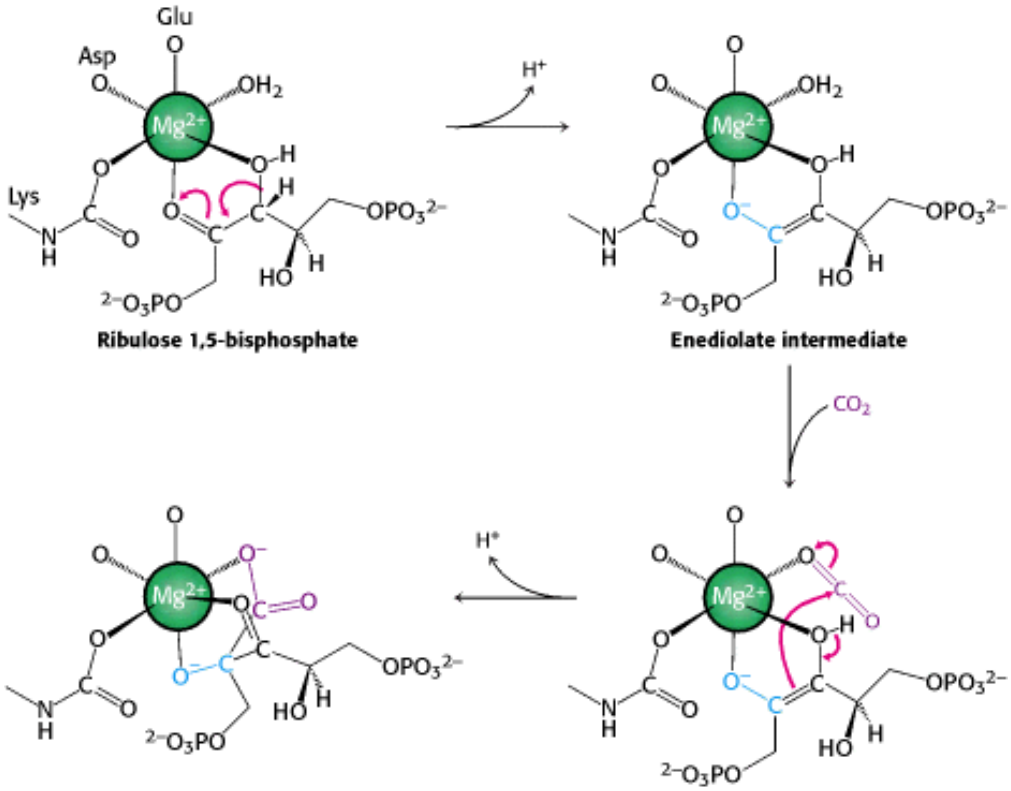


Figure 20.4. Role of the Magnesium Ion in the Rubisco Mechanism. Ribulose 1,5-bisphosphate binds to a magnesium ion that is linked to rubisco through a glutamate residue, an aspartate residue, and the lysine carbamate. The coordinated

ribulose 1,5-bisphosphate gives up a proton to form a reactive enediolate species that reacts with CO_2 to form a new carbon-carbon bond.

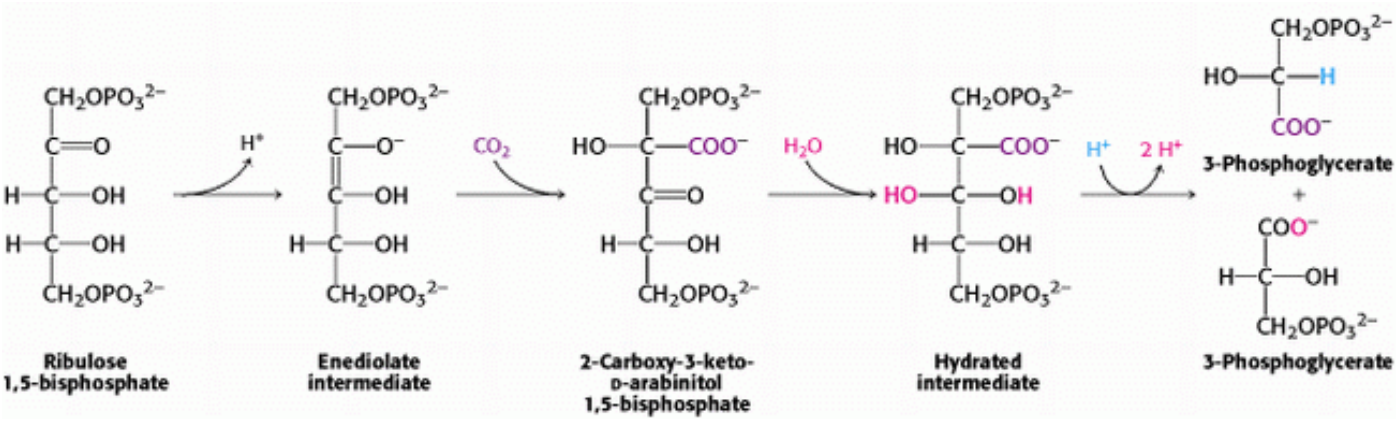


Figure 20.5. Formation of 3-Phosphoglycerate. The overall pathway for the conversion of ribulose 1,5 bisphosphate and CO_2 into two molecules of 3-phosphoglycerate. Although the free species are shown, these steps take place on the magnesium ion.

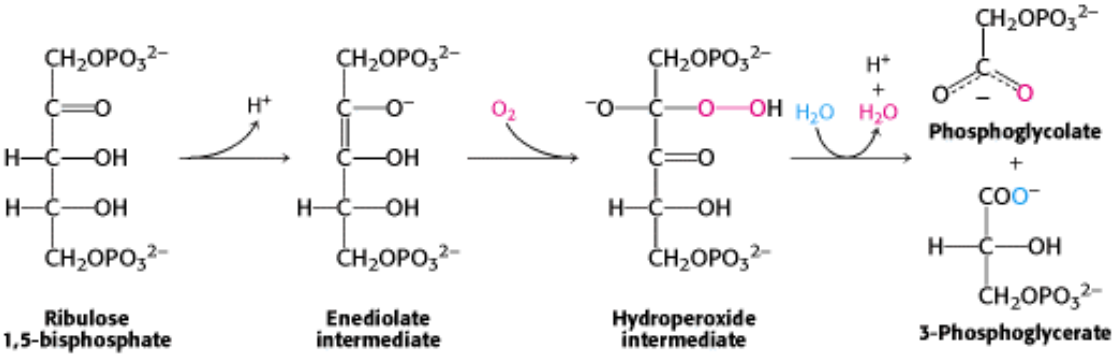


Figure 20.6. A Wasteful Side Reaction. The reactive enediolate intermediate on rubisco also reacts with molecular oxygen to form a hydroperoxide intermediate, which then proceeds to form one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate.

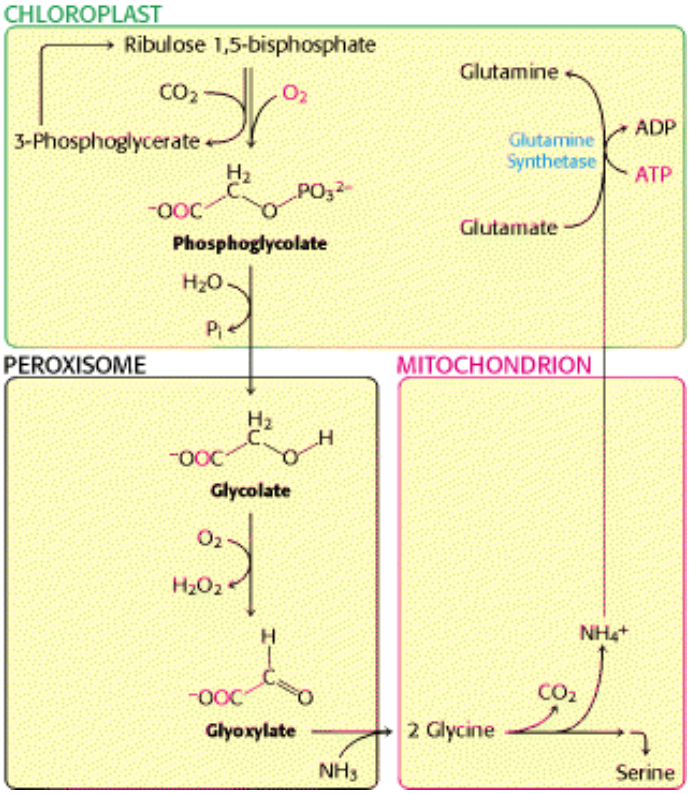


Figure 20.7. Photorespiratory Reactions. Phosphoglycolate is formed as a product of the oxygenase reaction in chloroplasts. After dephosphorylation, glycolate is transported into peroxisomes where it is converted into glyoxylate and then glycine. In mitochondria, two glycines are converted into serine, after losing a carbon as CO_2 and ammonia. The ammonia is salvaged in chloroplasts.

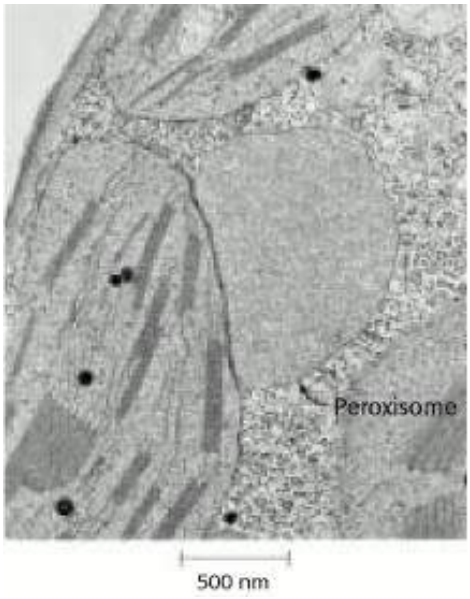


Figure 20.8. Electron Micrograph of a Peroxisome Nestled between Two Chloroplasts. [Courtesy of Dr. Sue Ellen Frederick.]

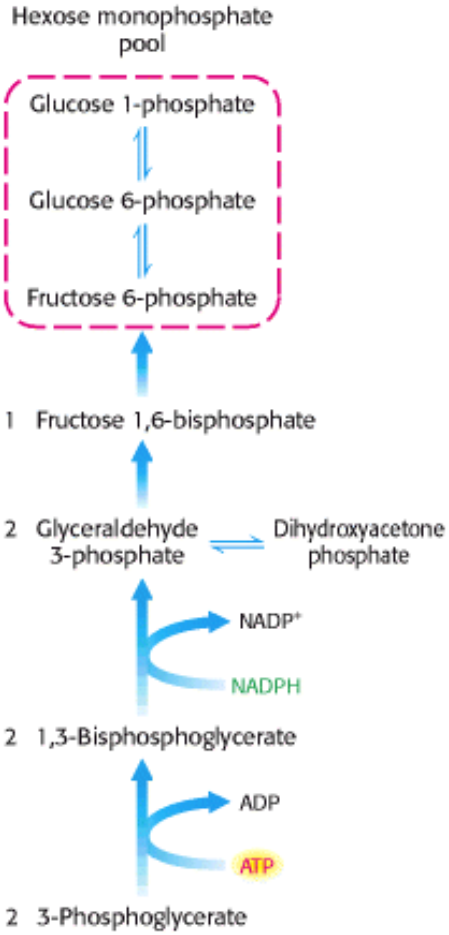


Figure 20.9. Hexose Phosphate Formation. 3-Phosphoglycerate is converted into fructose 6-phosphate in a pathway parallel to that of glyconeogenesis.

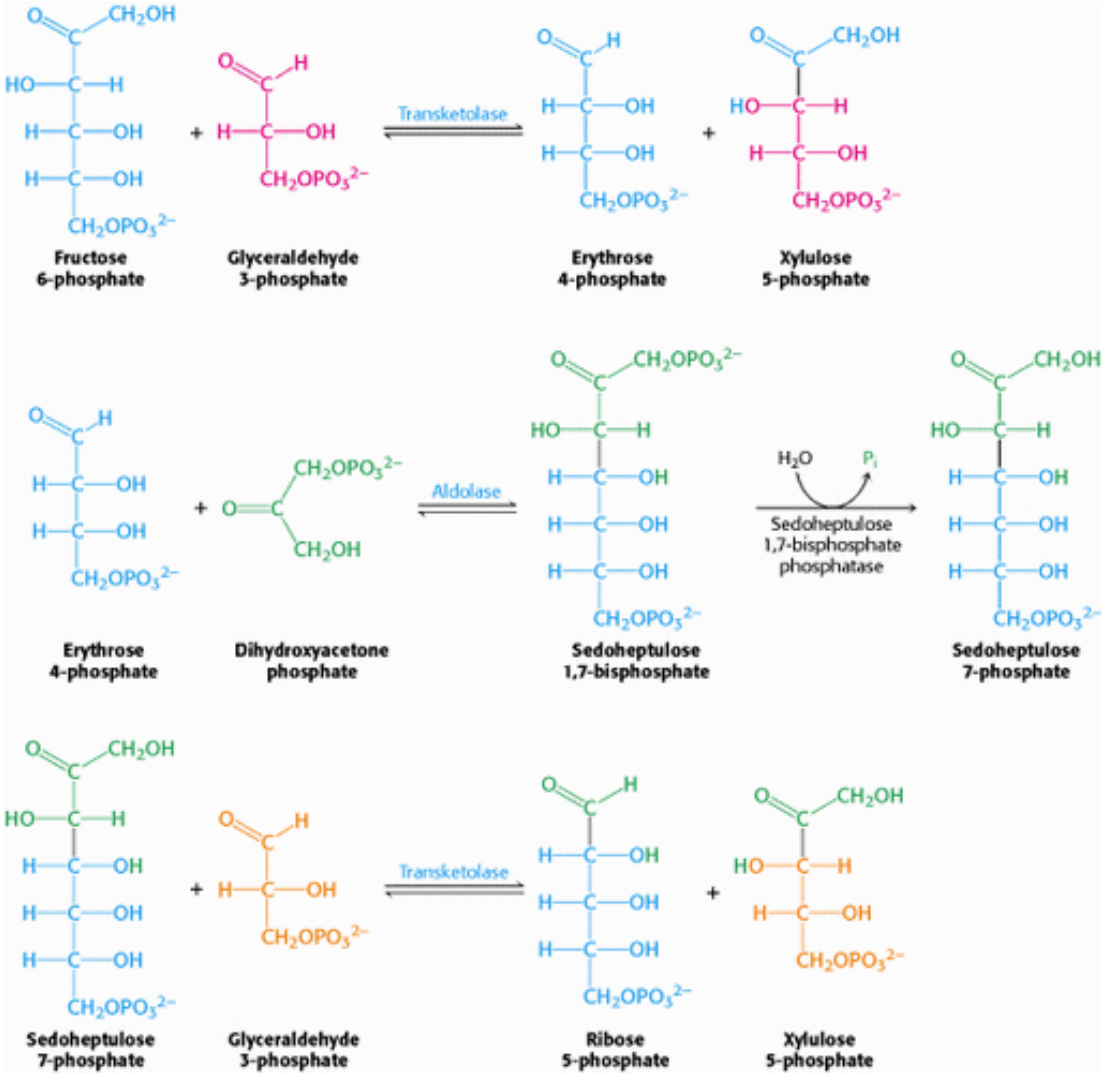


Figure 20.10. Formation of Five-Carbon Sugars. First, transketolase converts a six-carbon sugar and a three-carbon sugar into a four-carbon sugar and a five-carbon sugar. Then, aldolase combines the four-carbon product and a three-carbon sugar to form a seven-carbon sugar. Finally, this seven-carbon fragment combines with another three-carbon fragment to form two additional five-carbon sugars.

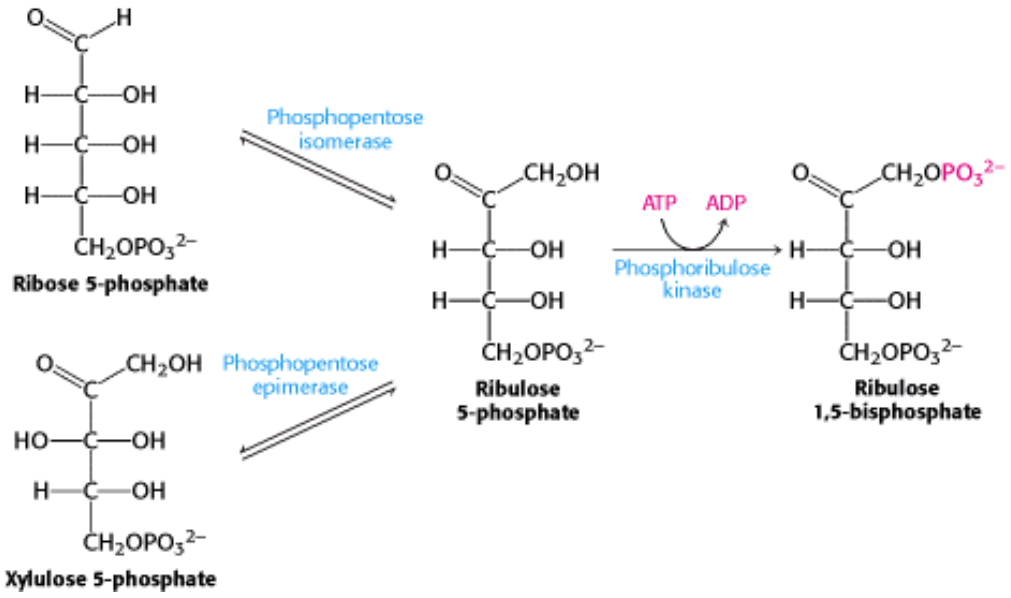


Figure 20.11. Regeneration of Ribulose 1,5-Bisphosphate. Both ribose 5-phosphate and xylulose 5-phosphate are converted into ribulose 5-phosphate, which is then phosphorylated to complete the regeneration of ribulose 1,5-bisphosphate.

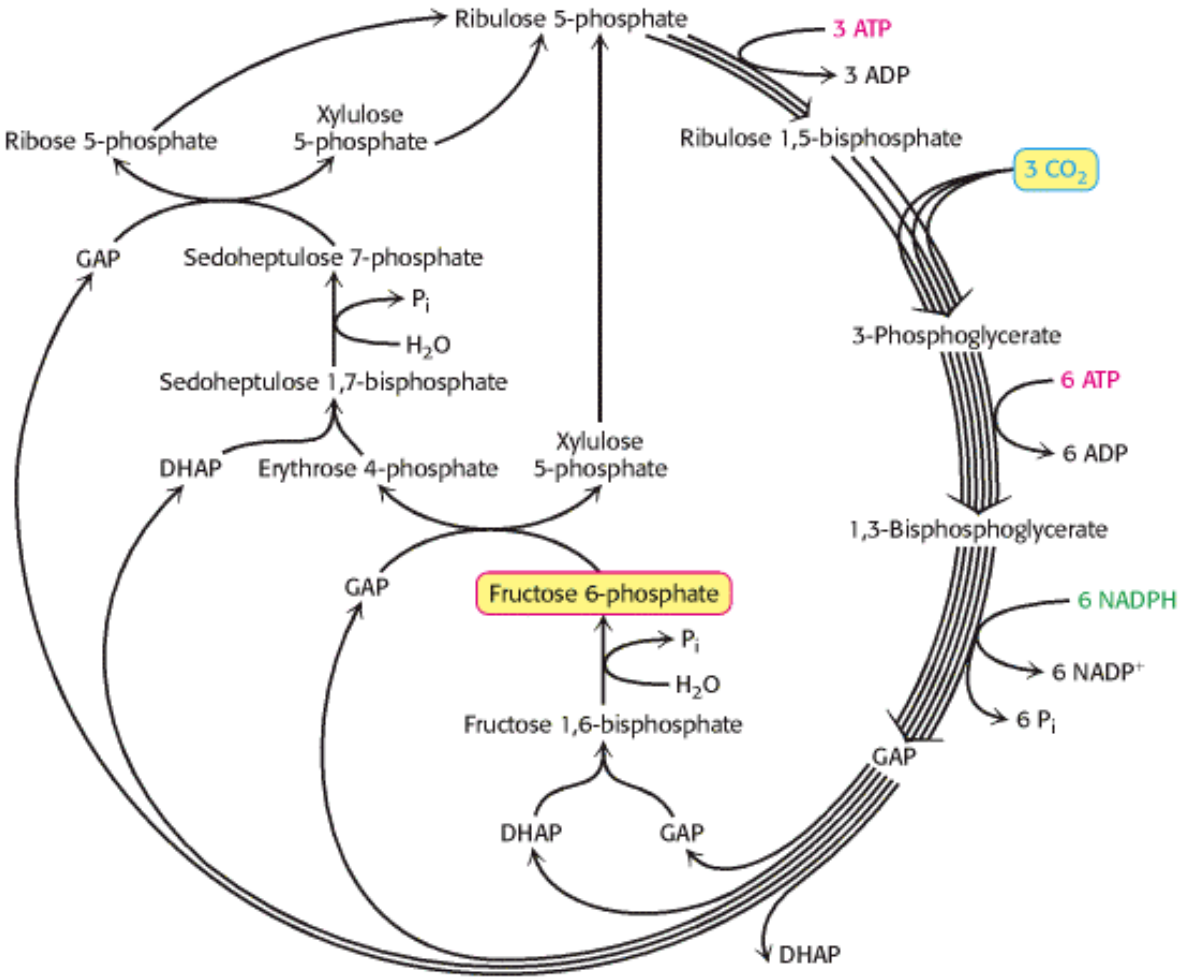


Figure 20.12. Calvin Cycle. The diagram shows the reactions necessary with the correct stoichiometry to convert three molecules of CO_2 into one molecule of DHAP. The cycle is not as simple as presented in Figure 20.1; rather, it entails many reactions that lead ultimately to the synthesis of glucose and the regeneration of ribulose 1,5-bisphosphate. [After J. R. Bowyer and R. C. Leegood. "Photosynthesis," in *Plant Biochemistry*, P. M. Dey and J. B. Harborne, Eds. (Academic Press, 1997), p. 85.]

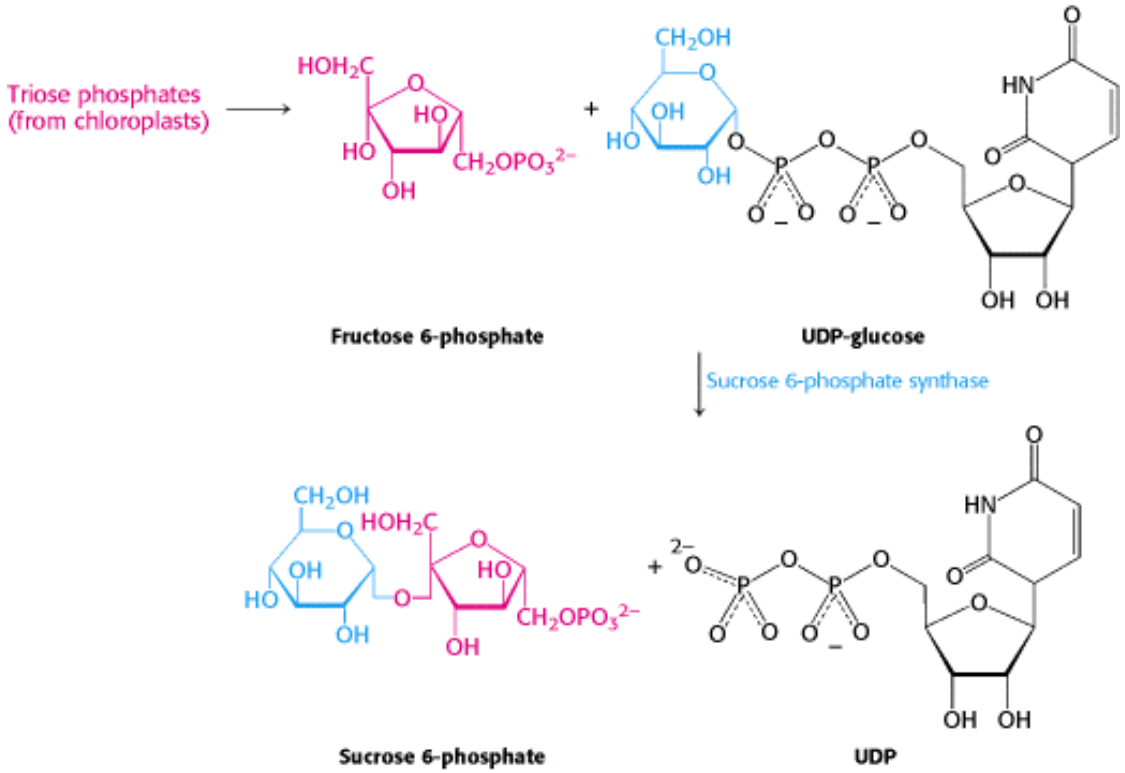


Figure 20.13. Synthesis of Sucrose. Sucrose 6-phosphate is formed by the reaction between fructose 6-phosphate and the activated intermediate uridine diphosphate glucose (UDP-glucose).

20.2. The Activity of the Calvin Cycle Depends on Environmental Conditions

Carbon dioxide assimilation by the Calvin cycle operates during the day, whereas carbohydrate degradation to yield energy takes place primarily at night. How are synthesis and degradation coordinately controlled? The light reactions lead to changes in the stroma—namely, an increase in pH and in Mg^{2+}, NADPH, and reduced ferredoxin concentration—all of which contribute to the activation of certain Calvin cycle enzymes (Figure 20.14).

20.2.1. Rubisco Is Activated by Light-Driven Changes in Proton and Magnesium Ion Concentrations

As stated earlier, the rate-limiting step in the Calvin cycle is the carboxylation of ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate. *The activity of rubisco increases markedly on illumination*. The addition of CO_2 to lysine 201 of rubisco to form the carbamate is essential for Mg^{2+} coordination and, hence, catalytic activity (Section 20.1.1). Carbamate formation is favored by alkaline pH and high concentrations of Mg^{2+} ion in the stroma, both of which are consequences of the light-driven pumping of protons from the stroma into the thylakoid space. Magnesium ion concentration rises because Mg^{2+} ions from the thylakoid space are released into the stroma to compensate for the influx of protons.

20.2.2. Thioredoxin Plays a Key Role in Regulating the Calvin Cycle

Light-driven reactions lead to electron transfer from water to ferredoxin and, eventually, to NADPH. Both reduced ferredoxin and NADPH regulate enzymes from the Calvin cycle. One key protein in these regulatory processes is *thioredoxin*, a 12-kd protein containing neighboring cysteine residues that cycle between a reduced sulfhydryl and an oxidized disulfide form (Figure 20.15). The reduced form of thioredoxin activates many biosynthetic enzymes by reducing disulfide bridges that control their activity and inhibits several degradative enzymes by the same means (Table

20.1). In chloroplasts, oxidized thioredoxin is reduced by ferredoxin in a reaction catalyzed by *ferredoxin-thioredoxin reductase*. This enzyme contains a 4Fe-4S cluster that couples two one-electron oxidations of reduced ferredoxin to the two-electron reduction of thioredoxin. Thus, *the activities of the light and dark reactions of photosynthesis are coordinated through electron transfer from reduced ferredoxin to thioredoxin and then to component enzymes containing regulatory disulfide bonds* (Figure 20.16). We shall return to thioredoxin when we consider the reduction of ribonucleotides (Section 25.3).

Other means of control also exist. For instance, phosphoribulose kinase and glyceraldehyde 3-phosphate dehydrogenase also are regulated by NADPH directly. In the dark, these enzymes associate with a small protein called CP12 to form a large complex in which the enzymes are inactivated. NADPH generated in the light reactions binds to this complex, leading to the release of the enzymes. Thus, the activity of these enzymes depends first on reduction by thioredoxin and then on the NADPH-mediated release from CP12.

20.2.3. The C_4 Pathway of Tropical Plants Accelerates Photosynthesis by Concentrating Carbon Dioxide

Recall that the oxygenase activity of rubisco increases more rapidly with temperature than does its carboxylase activity. How then do plants, such as sugar cane, that grow in hot climates prevent very high rates of wasteful photorespiration? Their solution to this problem is to achieve a high local concentration of CO_2 at the site of the Calvin cycle in their photosynthetic cells. The essence of this process, which was elucidated by M. D. Hatch and C. R. Slack, is that *four-carbon (C_4) compounds such as oxaloacetate and malate carry CO_2 from mesophyll cells, which are in contact with air, to bundle-sheath cells, which are the major sites of photosynthesis* (Figure 20.17). Decarboxylation of the four-carbon compound in a bundle-sheath cell maintains a high concentration of CO_2 at the site of the Calvin cycle. The three-carbon compound pyruvate returns to the mesophyll cell for another round of carboxylation.

The *C_4 pathway* for the transport of CO_2 starts in a mesophyll cell with the condensation of CO_2 and phosphoenolpyruvate to form *oxaloacetate*, in a reaction catalyzed by *phosphoenolpyruvate carboxylase*. In some species, oxaloacetate is converted into *malate* by an $NADP^+$-linked malate dehydrogenase. Malate goes into the bundle-sheath cell and is oxidatively decarboxylated within the chloroplasts by an $NADP^+$-linked malate dehydrogenase. The released CO_2 enters the Calvin cycle in the usual way by condensing with ribulose 1,5-bisphosphate. Pyruvate formed in this decarboxylation reaction returns to the mesophyll cell. Finally, phosphoenolpyruvate is formed from pyruvate by *pyruvate-P_i dikinase*.

The net reaction of this C_4 pathway is

$$CO_2\,(\text{in mesophyll cell}) + ATP + H_2O \longrightarrow CO_2\,(\text{in bundle-sheath cell}) + AMP + 2\,P_i + H^+$$

Thus, *the energetic equivalent of two ATP molecules is consumed in transporting CO_2 to the chloroplasts of the bundle-sheath cells*. In essence, this process is active transport: the pumping of CO_2 into the bundle-sheath cell is driven by the hydrolysis of one molecule of ATP to one molecule of AMP and two molecules of orthophosphate. The CO_2 concentration can be 20-fold as great in the bundle-sheath cells as in the mesophyll cells.

When the C_4 pathway and the Calvin cycle operate together, the net reaction is

$$6\,CO_2 + 30\,ATP + 12\,NADPH + 12\,H_2O \longrightarrow C_6H_{12}O_6 + 30\,ADP + 30\,P_i + 12\,NADP^+ + 18\,H^+$$

Note that 30 molecules of ATP are consumed per hexose molecule formed when the C_4 pathway delivers CO_2 to the Calvin cycle, in contrast with 18 molecules of ATP per hexose molecule in the absence of the C_4 pathway. The high concentration of CO_2 in the bundle-sheath cells of C_4 plants, which is due to the expenditure of the additional 12 molecules of ATP, is critical for their rapid photosynthetic rate, because CO_2 is limiting when light is abundant. A high CO_2 concentration also minimizes the energy loss caused by photorespiration.

Tropical plants with a C_4 pathway do little photorespiration because the high concentration of CO_2 in their bundle-sheath cells accelerates the carboxylase reaction relative to the oxygenase reaction. This effect is especially important at higher temperatures. The geographic distribution of plants having this pathway (C_4 plants) and those lacking it (C_3 plants) can now be understood in molecular terms. *C_4 plants* have the advantage in a hot environment and under high illumination, which accounts for their prevalence in the tropics. *C_3 plants*, which consume only 18 molecules of ATP per hexose molecule formed in the absence of photorespiration (compared with 30 molecules of ATP for C_4 plants), are more efficient at temperatures of less than about 28°C, and so they predominate in temperate environments.

Rubisco is found in bacteria, eukaryotes, and even archaea, though other photosynthetic components have not been found in archaea. Thus, rubisco emerged early in evolution, when the atmosphere was rich in CO_2 and almost devoid of O_2. The enzyme was not originally selected to operate in an environment like the present one, which is almost devoid of CO_2 and rich in O_2. Photorespiration became significant about 60 million years ago, when the CO_2 concentration fell to present levels. The C_4 pathway is thought to have evolved in response to this selective pressure no more than 30 million years ago and possibly as recently as 7 million years ago. It is interesting to note that none of the enzymes are unique to C_4 plants, suggesting that this pathway was created using existing enzymes.

20.2.4. Crassulacean Acid Metabolism Permits Growth in Arid Ecosystems

Crassulacean acid metabolism (CAM) is yet another adaptation to increase the efficiency of the Calvin cycle. Crassulacean acid metabolism, named after the genus *Crassulacea* (the succulents), is a response to drought as well as warm conditions. In CAM plants, the stomata of the leaves are closed in the heat of the day to prevent water loss (Figure 20.18). As a consequence, CO_2 cannot be absorbed during the daylight hours when it is needed for glucose synthesis. When the stomata open at the cooler temperatures of night, CO_2 is fixed by the C_4 pathway into malate, which is stored in vacuoles. During the day, malate is decarboxylated and the CO_2 becomes available to the Calvin cycle. In contrast with C_4 plants, CO_2 accumulation is separated from CO_2 utilization temporally in CAM plants rather than spatially.

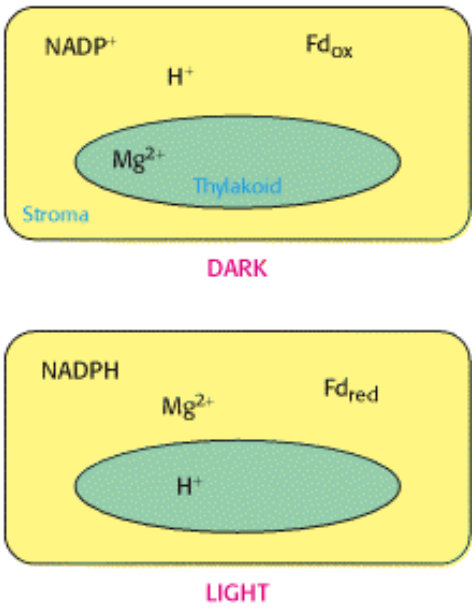


Figure 20.14. Light Regulation of the Calvin Cycle. The light reactions of photosynthesis transfer electrons out of the thylakoid lumen into the stroma and they transfer protons from the stroma into the thylakoid lumen. As a consequence of these processes, the concentrations of NADPH, reduced ferredoxin (Fd), and Mg^{2+} in the stroma are higher in the light than in the dark, whereas the concentration of H^+ is lower in the dark. Each of these concentration changes helps couple the Calvin cycle reactions to the light reactions.

Figure 20.15. Thioredoxin. The oxidized form of thioredoxin contains a disulfide bond. When thioredoxin is reduced by reduced ferredoxin, the disulfide bond is converted into two free sulfhydryl groups. Reduced thioredoxin can cleave disulfide bonds in enzymes, activating certain Calvin cycle enzymes and inactivating some degradative enzymes.

Table 20.1. Enzymes regulated by thioredoxin

Enzyme	Pathway
Rubisco	Carbon fixation in the Calvin cycle
Fructose 1,6-bisphosphatase	Gluconeogenesis
Glyceraldehyde 3-phosphate dehydrogenase	Calvin cycle, gluconeogenesis, glycolysis
Sedoheptulose bisphosphatase	Calvin cycle

Glucose 6-phosphate dehydrogenase	Pentose phosphate pathway
Phenylalanine ammonia lyase	Lignin synthesis
Ribulose 5′-phosphate kinase	Calvin cycle
$NADP^+$-malate dehydrogenase	C_4 pathway

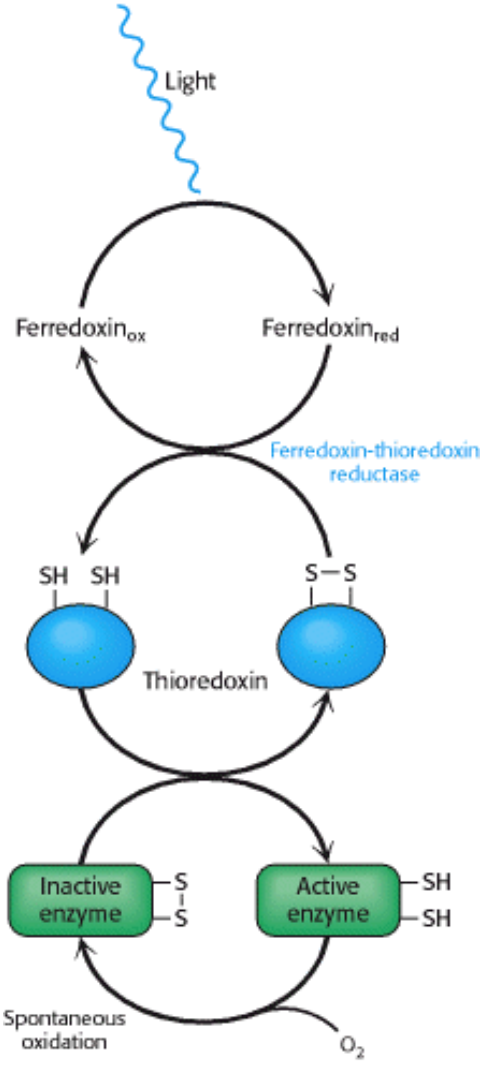


Figure 20.16. Enzyme Activation by Thioredoxin. Reduced thioredoxin activates certain Calvin cycle enzymes by cleaving regulatory disulfide bonds.

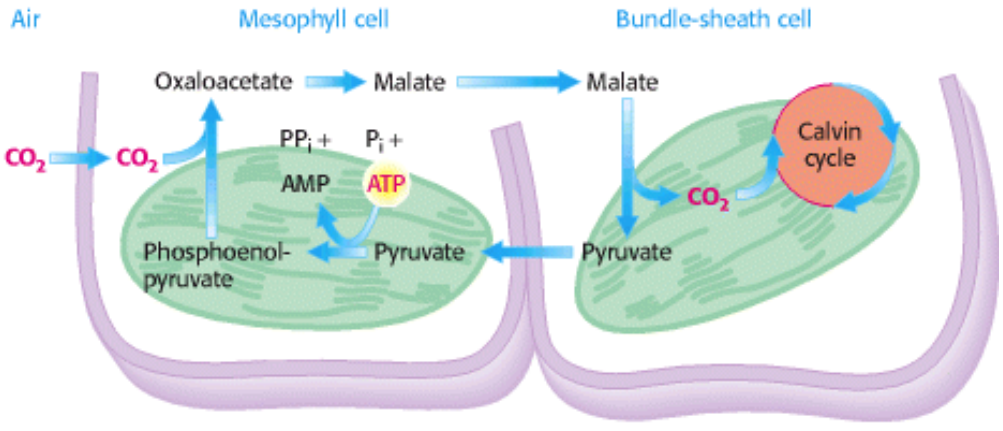


Figure 20.17. C_4 Pathway. Carbon dioxide is concentrated in bundle-sheath cells by the expenditure of ATP in mesophyll cells.

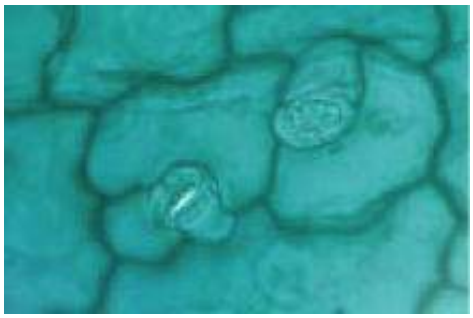


Figure 20.18. Electron Micrograph of an Open Stoma and a Closed Stoma. [Herb Charles Ohlmeyer/Fran Heyl Associates.]

20.3 the Pentose Phosphate Pathway Generates NADPH and Synthesizes Five-Carbon Sugars

The pentose phosphate pathway meets the need of all organisms for a source of NADPH to use in reductive biosynthesis (Table 20.2). This pathway consists of two phases: the oxidative generation of NADPH and the nonoxidative interconversion of sugars (Figure 20.19). In the oxidative phase, NADPH is generated when glucose 6-phosphate is oxidized to ribose 5-phosphate. This five-carbon sugar and its derivatives are components of RNA and DNA, as well as ATP, NADH, FAD, and coenzyme A.

$$\text{Glucose 6-phosphate} + 2\,\text{NADP}^+ + \text{H}_2\text{O} \longrightarrow \text{ribose 5-phosphate} + 2\,\text{NADPH} + 2\,\text{H}^+ + \text{CO}_2$$

In the nonoxidative phase, the pathway catalyzes the interconversion of three-, four-, five-, six-, and seven-carbon sugars in a series of nonoxidative reactions that can result in the synthesis of five-carbon sugars for nucleotide biosynthesis or the conversion of excess five-carbon sugars into intermediates of the glycolytic pathway. All these reactions take place in the cytosol. These interconversions rely on the same reactions that lead to the regeneration of ribulose 1,5-bisphosphate in the Calvin cycle.

20.3.1. Two Molecules of NADPH Are Generated in the Conversion of Glucose 6-phosphate into Ribulose 5-phosphate

The oxidative phase of the pentose phosphate pathway starts with the dehydrogenation of glucose 6-phosphate at carbon 1, a reaction catalyzed by *glucose 6-phosphate dehydrogenase* (Figure 20.20). This enzyme is highly specific for $NADP^+$; the K_M for NAD^+ is about a thousand times as great as that for $NADP^+$. The product is *6-phosphoglucono-δ-lactone*, which is an intramolecular ester between the C-1 carboxyl group and the C-5 hydroxyl group. The next step is the hydrolysis of 6-phosphoglucono-δ-lactone by a specific *lactonase* to give *6-phosphogluconate*. This six-carbon sugar is then oxidatively decarboxylated by *6-phosphogluconate dehydrogenase* to yield *ribulose 5-phosphate*. $NADP^+$ is again the electron acceptor. The final step in the synthesis of ribose 5-phosphate is the isomerization of ribulose 5-phosphate by phosphopentose isomerase (see Figure 20.11)

20.3.2. The Pentose Phosphate Pathway and Glycolysis Are Linked by Transketolase

and Transaldolase

The preceding reactions yield two molecules of NADPH and one molecule of ribose 5-phosphate for each molecule of glucose 6-phosphate oxidized. However, many cells need NADPH for reductive biosyntheses much more than they need ribose 5-phosphate for incorporation into nucleotides and nucleic acids. In these cases, ribose 5-phosphate is converted into glyceraldehyde 3-phosphate and fructose 6-phosphate by *transketolase* and *transaldolase. These enzymes create a reversible link between the pentose phosphate pathway and glycolysis by catalyzing these three successive reactions.*

$$C_5 + C_5 \xrightleftharpoons{\text{Transketolase}} C_3 + C_7$$

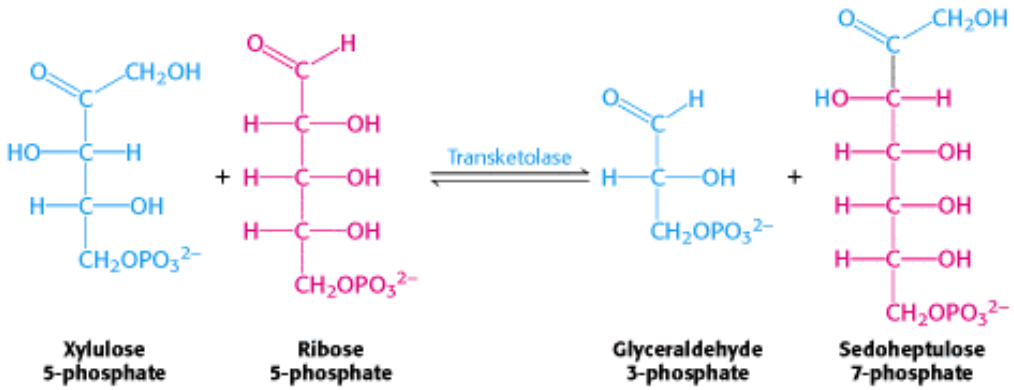
$$C_3 + C_7 \xrightleftharpoons{\text{Transketolase}} C_6 + C_4$$

$$C_4 + C_5 \xrightleftharpoons{\text{Transketolase}} C_6 + C_3$$

The net result of these reactions is the *formation of two hexoses and one triose from three pentoses:*

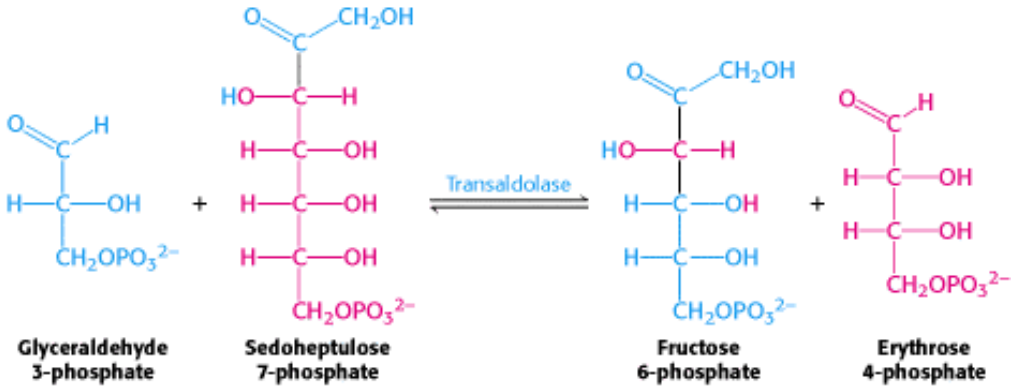
$$3\,C_5 \rightleftharpoons 2\,C_6 + C_3$$

The first of the three reactions linking the pentose phosphate pathway and glycolysis is the formation of *glyceraldehyde 3-phosphate* and *sedohep-tulose 7-phosphate* from two pentoses.



The donor of the two-carbon unit in this reaction is xylulose 5-phosphate, an epimer of ribulose 5-phosphate. A ketose is a substrate of transketolase only if its hydroxyl group at C-3 has the configuration of xylulose rather than ribulose. Ribulose 5-phosphate is converted into the appropriate epimer for the transketolase reaction by *phosphopentose epimerase* (see Figure 20.11) in the reverse reaction of that which occurs in the Calvin cycle.

Glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate generated by the transketolase then react to form *fructose 6-phosphate* and *erythrose 4-phosphate.*



This synthesis of a four-carbon sugar and a six-carbon sugar is catalyzed by *transaldolase*.

In the third reaction, transketolase catalyzes the synthesis of *fructose 6-phosphate* and *glyceraldehyde 3-phosphate* from erythrose 4-phosphate and xylulose 5-phosphate.

The sum of these reactions is

$$\text{2 Xylulose 5-phosphate} + \text{ribose 5-phosphate} \rightleftharpoons \text{2 fructose 6-phosphate} + \text{glyceraldehyde 3-phosphate}$$

Xylulose 5-phosphate can be formed from ribose 5-phosphate by the sequential action of phosphopentose isomerase and phosphopentose epimerase, and so the net reaction starting from ribose 5-phosphate is

$$\text{3 Ribose 5-phosphate} \rightleftharpoons \text{2 fructose 6-phosphate} + \text{glyceraldehyde 3-phosphate}$$

Thus, *excess ribose 5-phosphate formed by the pentose phosphate pathway can be completely converted into glycolytic intermediates*. Moreover, any ribose ingested in the diet can be processed into glycolytic intermediates by this pathway. It is evident that the carbon skeletons of sugars can be extensively rearranged to meet physiologic needs (Table 20.3).

20.3.3. Transketolase and Transaldolase Stabilize Carbanionic Intermediates by Different Mechanisms

The reactions catalyzed by transketolase and transaldolase are distinct yet similar in many ways. One difference is that transketolase transfers a two-carbon unit, whereas transaldolase transfers a three-carbon unit. Each of these units is transiently attached to the enzyme in the course of the reaction. In transketolase, the site of addition of the unit is the

thiazole ring of the required coenzyme thiamine pyrophosphate. Transketolase is homologous to the E_1 subunit of the pyruvate dehydrogenase complex (Section 17.1.1) and the reaction mechanism is similar (Figure 20.21).

The C-2 carbon atom of bound TPP readily ionizes to give a *carbanion*. The negatively charged carbon atom of this reactive intermediate attacks the carbonyl group of the ketose substrate. The resulting addition compound releases the aldose product to yield an *activated glycoaldehyde unit*. The positively charged nitrogen atom in the thiazole ring acts as an *electron sink* in the development of this activated intermediate. The carbonyl group of a suitable aldose acceptor then condenses with the activated glycoaldehyde unit to form a new ketose, which is released from the enzyme.

Transaldolase transfers a three-carbon *dihydroxyacetone* unit from a ketose donor to an aldose acceptor. Transaldolase, in contrast with transketolase, does not contain a prosthetic group. Rather, *a Schiff base is formed between the carbonyl group of the ketose substrate and the* ε*-amino group of a lysine residue at the active site of the enzyme* (Figure 20.22). This kind of covalent enzyme-substrate intermediate is like that formed in fructose 1,6-bisphosphate aldolase in the glycolytic pathway (Section 16.1.3) and, indeed, the enzymes are homologous. The Schiff base becomes protonated, the bond between C-3 and C-4 is split, and an aldose is released. The negative charge on the Schiff-base carbanion moiety is stabilized by resonance. The positively charged nitrogen atom of the protonated Schiff base acts as an electron sink. The Schiff-base adduct is stable until a suitable aldose becomes bound. The dihydroxyacetone moiety then reacts with the carbonyl group of the aldose. The ketose product is released by hydrolysis of the Schiff base. The nitrogen atom of the protonated Schiff base plays the same role in transaldolase as the thiazole-ring nitrogen atom does in transketolase. In each enzyme, a group within an intermediate reacts like a carbanion in attacking a carbonyl group to form a new carbon-carbon bond. In each case, the charge on the carbanion is stabilized by resonance (Figure 20.23).

Table 20.2. Pathways requiring NADPH

Synthesis
Fatty acid biosynthesis
Cholesterol biosynthesis
Neurotransmitter biosynthesis
Nucleotide biosynthesis
Detoxification
Reduction of oxidized glutathione
Cytochrome P450 monooxygenases

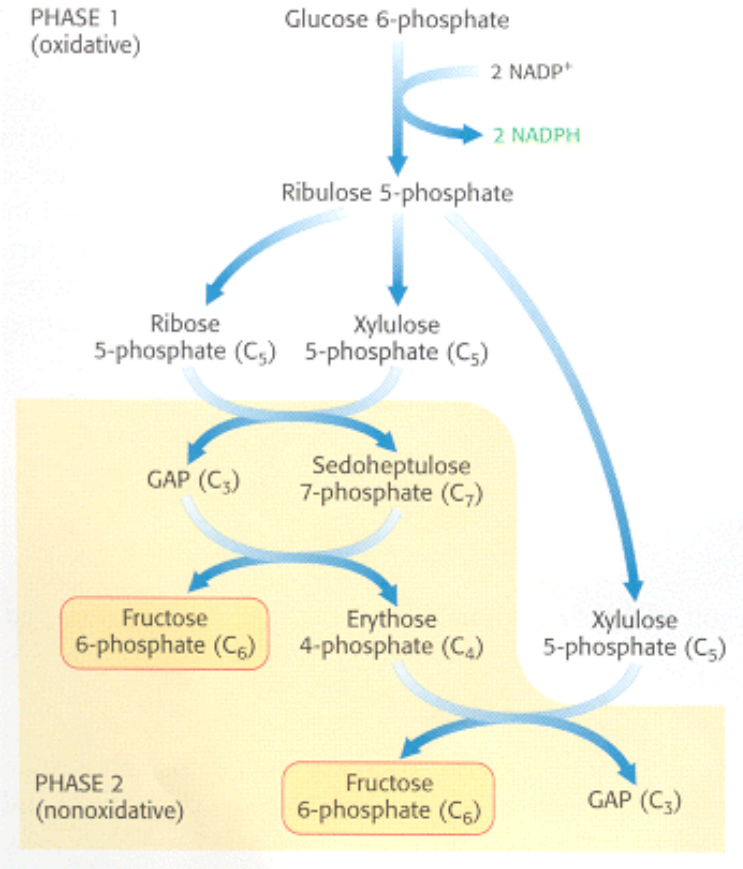


Figure 20.19. Pentose Phosphate Pathway. The pathway consists of (1) an oxidative phase that generates NADPH and (2) a nonoxidative phase that interconverts phosphorylated sugars.

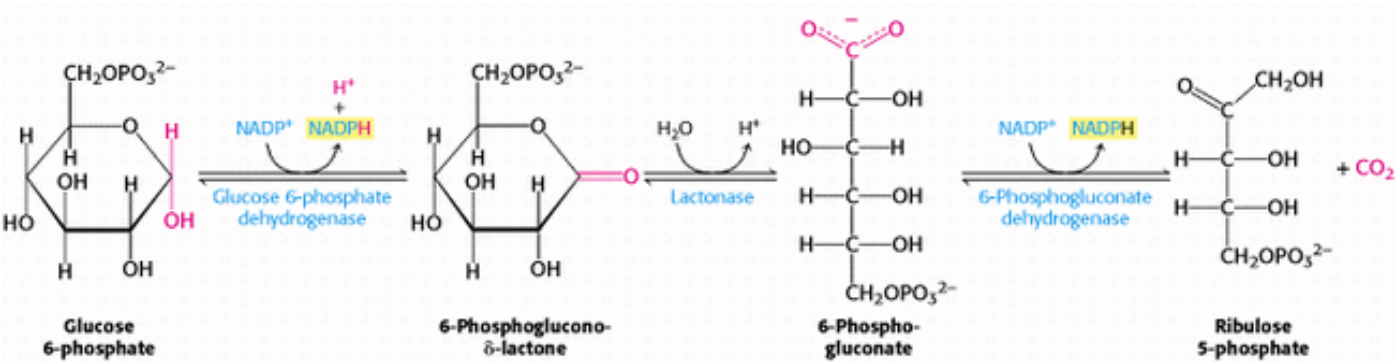


Figure 20.20. Oxidative Phase of the Pentose Phosphate Pathway. Glucose 6-phosphate is oxidized to 6-phosphoglucono-δ-lactone to generate one molecule of NADPH. The lactone product is hydrolyzed to 6-phosphogluconate, which is oxidatively decarboxylated to ribulose 5-phosphate with the generation of a second molecule of NADPH.

Table 20.3. Pentose phosphate pathway

Reaction	Enzyme
Oxidative phase	
Glucose 6-phosphate + $NADP^+ \rightarrow$ 6-phosphoglucono-δ-lactone + NADPH + H^+	Glucose 6-phosphate dehydrogenase
6-Phosphoglucono-δ-lactone + $H_2O \rightarrow$ 6-phosphogluconate + H^+	Lactonase
6-Phosphogluconate + $NADP^+ \rightarrow$ ribulose 5-phosphate + CO_2 + NADPH	6-Phosphogluconate dehydrogenase
Nonoxidative Phase	
Ribulose 5-phosphate ⇌ ribose 5-phosphate	Phosphopentose isomerase
Ribulose 5-phosphate ⇌ xylulose 5-phosphate	Phosphopentose epimerase
Xylulose 5-phosphate + ribose 5-phosphate ⇌ sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate	Transketolase
Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate ⇌ fructose 6-phosphate + erythrose 4-phosphate	Transaldolase
Xylulose 5-phosphate + erythrose 4-phosphate ⇌ fructose 6-phosphate + glyceraldehyde 3-phosphate	Transketolase

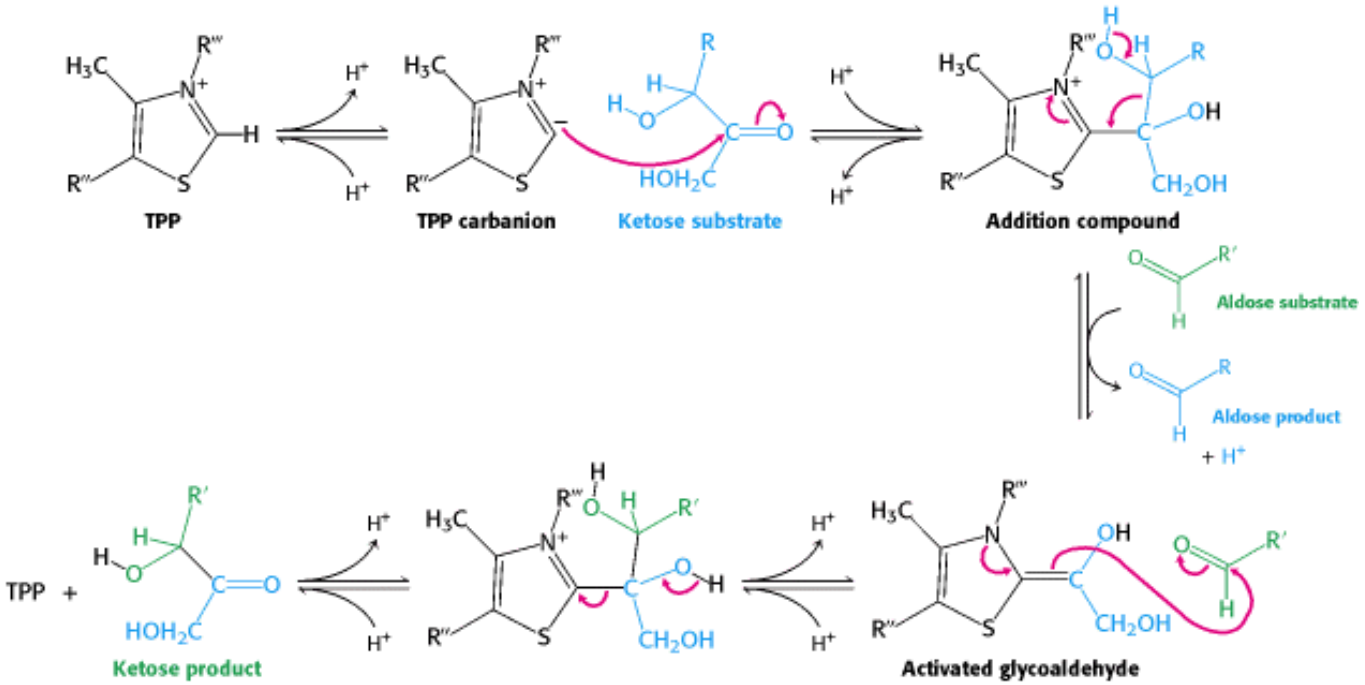


Figure 20.21. Transketolase Mechanism. The carbanion of thiamine pyrophosphate (TPP) attacks the ketose substrate. Cleavage of a carbon-carbon bond frees the aldose product and leaves a two-carbon fragment joined to TPP. This activated glycoaldehyde intermediate attacks the aldose substrate to form a new carbon-carbon bond. The ketose product is released, freeing the TPP for the next reaction cycle.

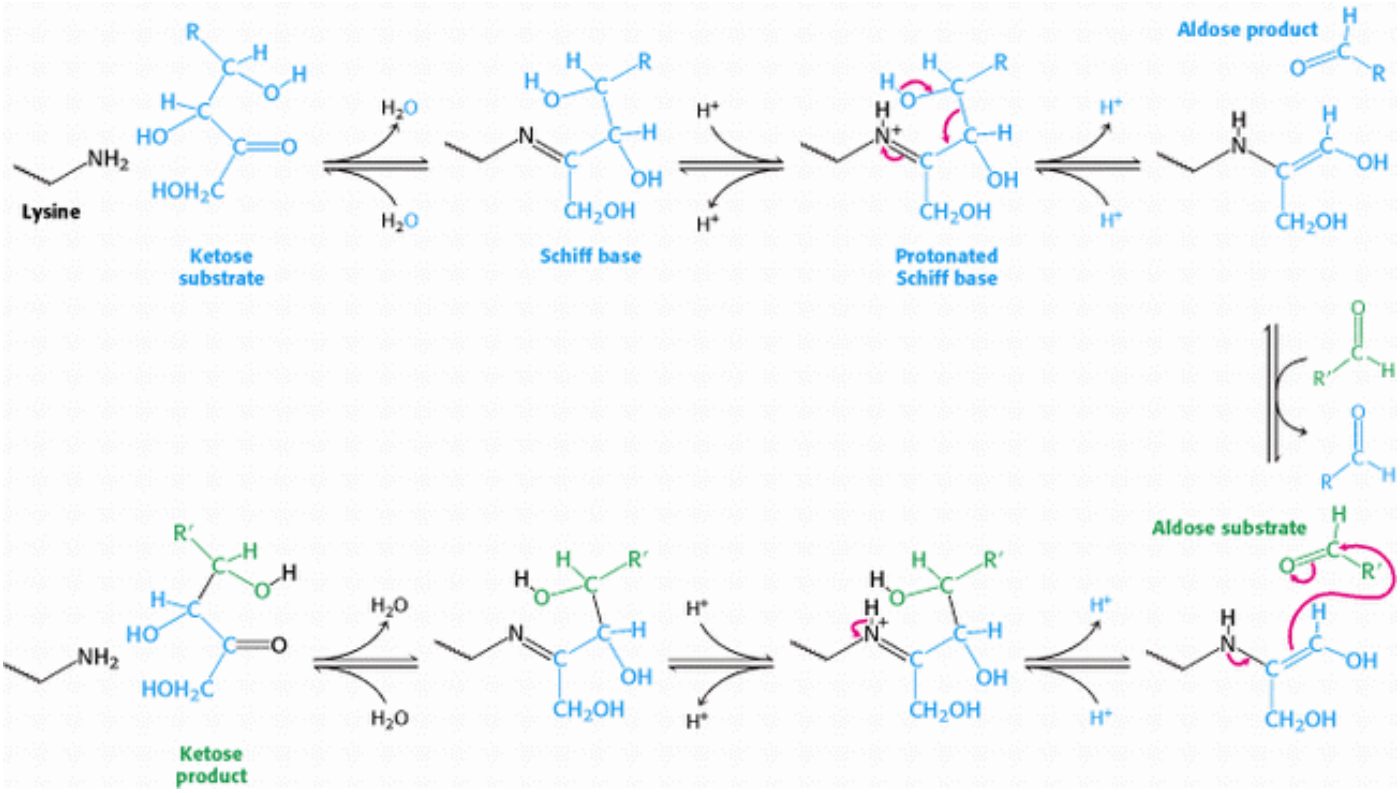


Figure 20.22. Transaldolase Mechanism. The reaction begins with the formation of a Schiff base between a lysine residue in transaldolase and the ketose substrate. Protonation of the Schiff base leads to release of the aldose product, leaving a three-carbon fragment attached to the lysine residue. This intermediate adds to the aldose substrate to form a new carbon-carbon bond. The reaction cycle is completed by release of the ketose product from the lysine side chain.

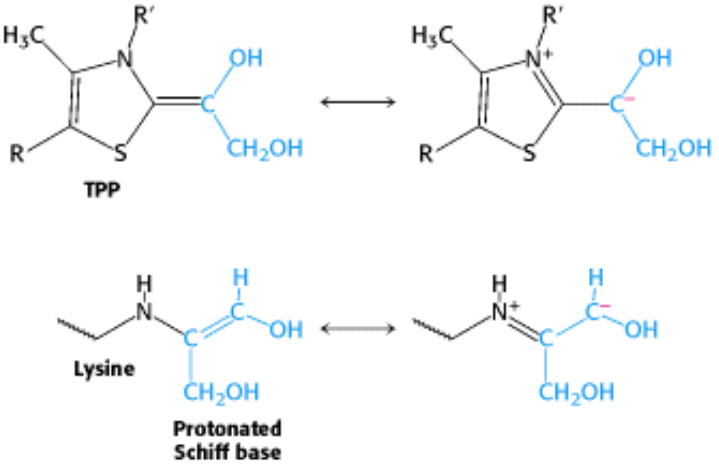


Figure 20.23. Carbanion Intermediates. For transketolase and transaldolase, a carbanion intermediate is stabilized by resonance. In transketolase, TPP stabilizes this intermediate; in transaldolase, a protonated Schiff base plays this role.

20.4. The Metabolism of Glucose 6-Phosphate by the Pentose Phosphate Pathway Is Coordinated with Glycolysis

Conceptual Insights, Overview of Carbohydrate and Fatty Acid Metabolism. View this media module to gain a "bigger picture" understanding of the roles of the pentose phosphate pathway in the context of other metabolic pathways (glycolysis, citric acid cycle, glycogen and fatty acid metabolism).

Glucose 6-phosphate is metabolized by both the glycolytic pathway (Chapter 16) and the pentose phosphate pathway. How is the processing of this important metabolite partitioned between these two metabolic routes? The cytoplasmic concentration of $NADP^+$ plays a key role in determining the fate of glucose 6-phosphate.

20.4.1. The Rate of the Pentose Phosphate Pathway Is Controlled by the Level of $NADP^+$

The first reaction in the oxidative branch of the pentose phosphate pathway, the dehydrogenation of glucose 6-phosphate, is essentially irreversible. In fact, this reaction is rate limiting under physiological conditions and serves as the control site. The most important regulatory factor is the level of $NADP^+$, the electron acceptor in the oxidation of glucose 6-phosphate to 6-phosphoglucono-δ-lactone. The inhibitory effect of low levels of $NADP^+$ is exacerbated by the fact that NADPH competes with $NADP^+$ in binding to the enzyme. The ratio of $NADP^+$ to NADPH in the cytosol of a liver cell from a well-fed rat is about 0.014, several orders of magnitude lower than the ratio of NAD^+ to NADH, which is 700 under the same conditions. The marked effect of the $NADP^+$ level on the rate of the oxidative phase ensures that NADPH generation is tightly coupled to its utilization in reductive biosyntheses. The nonoxidative phase of the pentose phosphate pathway is controlled primarily by the availability of substrates.

20.4.2. The Flow of Glucose 6-phosphate Depends on the Need for NADPH, Ribose 5-phosphate, and ATP

We can grasp the intricate interplay between glycolysis and the pentose phosphate pathway by examining the metabolism of glucose 6-phosphate in four different metabolic situations (Figure 20.24).

Mode 1. *Much more ribose 5-phosphate than NADPH is required*. For example, rapidly dividing cells need ribose 5-phosphate for the synthesis of nucleotide precursors of DNA. Most of the glucose 6-phosphate is converted into fructose 6-phosphate and glyceraldehyde 3-phosphate by the glycolytic pathway. Transaldolase and transketolase then convert two molecules of fructose 6-phosphate and one molecule of glyceraldehyde 3-phosphate into three molecules of ribose 5-phosphate by a reversal of the reactions described earlier. The stoichiometry of mode 1 is

$$5\ \text{Glucose 6-phosphate} + \text{ATP} \longrightarrow 6\ \text{ribose 5-phosphate} + \text{ADP} + \text{H}^+$$

Mode 2. *The needs for NADPH and ribose 5-phosphate are balanced*. The predominant reaction under these conditions is the formation of two molecules of NADPH and one molecule of ribose 5-phosphate from one molecule of glucose 6-phosphate in the oxidative phase of the pentose phosphate pathway. The stoichiometry of mode 2 is

$$\text{Glucose 6-phosphate} + 2\,\text{NADP}^+ + \text{H}_2\text{O} \longrightarrow \text{ribose 5-phosphate} + 2\,\text{NADPH} + 2\,\text{H}^+ + \text{CO}_2$$

Mode 3. *Much more NADPH than ribose 5-phosphate is required*. For example, adipose tissue requires a high level of NADPH for the synthesis of fatty acids (Table 20.4). In this case, glucose 6-phosphate is completely oxidized to CO_2. Three groups of reactions are active in this situation. First, the oxidative phase of the pentose phosphate pathway forms two molecules of NADPH and one molecule of ribose 5-phosphate. Then, ribose 5-phosphate is converted into fructose 6-phosphate and glyceraldehyde 3-phosphate by transketolase and transaldolase. Finally, glucose 6-phosphate is resynthesized from fructose 6-phosphate and glyceraldehyde 3-phosphate by the gluconeogenic pathway. The stoichiometries of these three sets of reactions are

$$6\ \text{Glucose 6-phosphate} + 12\ NADP^+ + 6\ H_2O \longrightarrow 6\ \text{ribose 5-phosphate} + 12\ NADPH + 12\ H^+ + 6\ CO_2$$

$$6\ \text{Ribose 5-phosphate} \longrightarrow 4\ \text{fructose 6-phosphate} + 2\ \text{glyceraldehyde 3-phosphate}$$

$$4\ \text{Fructose 6-phosphate} + 2\ \text{glyceraldehyde 3-phosphate} + H_2O \longrightarrow 5\ \text{glucose 6-phosphate} + P_i$$

The sum of the mode 3 reactions is

$$\text{Glucose 6-phosphate} + 12\ NADP^+ + 7\ H_2O \longrightarrow 6\ CO_2 + 12\ NADPH + 12\ H^+ + P_i$$

Thus, *the equivalent of glucose 6-phosphate can be completely oxidized to CO_2 with the concomitant generation of NADPH*. In essence, ribose 5-phosphate produced by the pentose phosphate pathway is recycled into glucose 6-phosphate by transketolase, transaldolase, and some of the enzymes of the gluconeogenic pathway.

Mode 4. *Both NADPH and ATP are required*. Alternatively, ribose 5-phosphate formed by the oxidative phase of the pentose phosphate pathway can be converted into pyruvate. Fructose 6-phosphate and glyceraldehyde 3-phosphate derived from ribose 5-phosphate enter the glycolytic pathway rather than reverting to glucose 6-phosphate. In this mode, *ATP and NADPH are concomitantly generated, and five of the six carbons of glucose 6-phosphate emerge in pyruvate*.

$$3\ \text{Glucose 6-phosphate} + 6\ NADP^+ + 5\ NAD^+ + 5\ P_i + 8\ ADP \longrightarrow 5\ \text{pyruvate} + 3\ CO_2 + 6\ NADPH + 5\ NADH + 8\ ATP + 2\ H_2O + 8\ H^+$$

Pyruvate formed by these reactions can be oxidized to generate more ATP or it can be used as a building block in a variety of biosyntheses.

20.4.3. Through the Looking Glass: The Calvin Cycle and the Pentose Phosphate Pathway

The complexities of the Calvin cycle and the pentose phosphate pathway are easier to comprehend if we consider them mirror images of each other. The Calvin cycle begins with the fixation of CO_2 and goes on to use NADPH in the synthesis of glucose. The pentose phosphate pathway begins with the oxidation of a glucose-derived carbon atom to CO_2

and concomitantly generates NADPH. The regeneration phase of the Calvin cycle converts C_6 and C_3 molecules back into the starting material—the C_5 molecule ribulose 1,5-bisphosphate. The pentose phosphate pathway converts a C_5 molecule, ribose 5-phosphate, into C_6 and C_3 intermediates of the glycolytic pathway. Not surprisingly, in photosynthetic organisms, many enzymes are common to the two pathways. We see the economy of evolution: the use of identical enzymes for similar reactions with different ends.

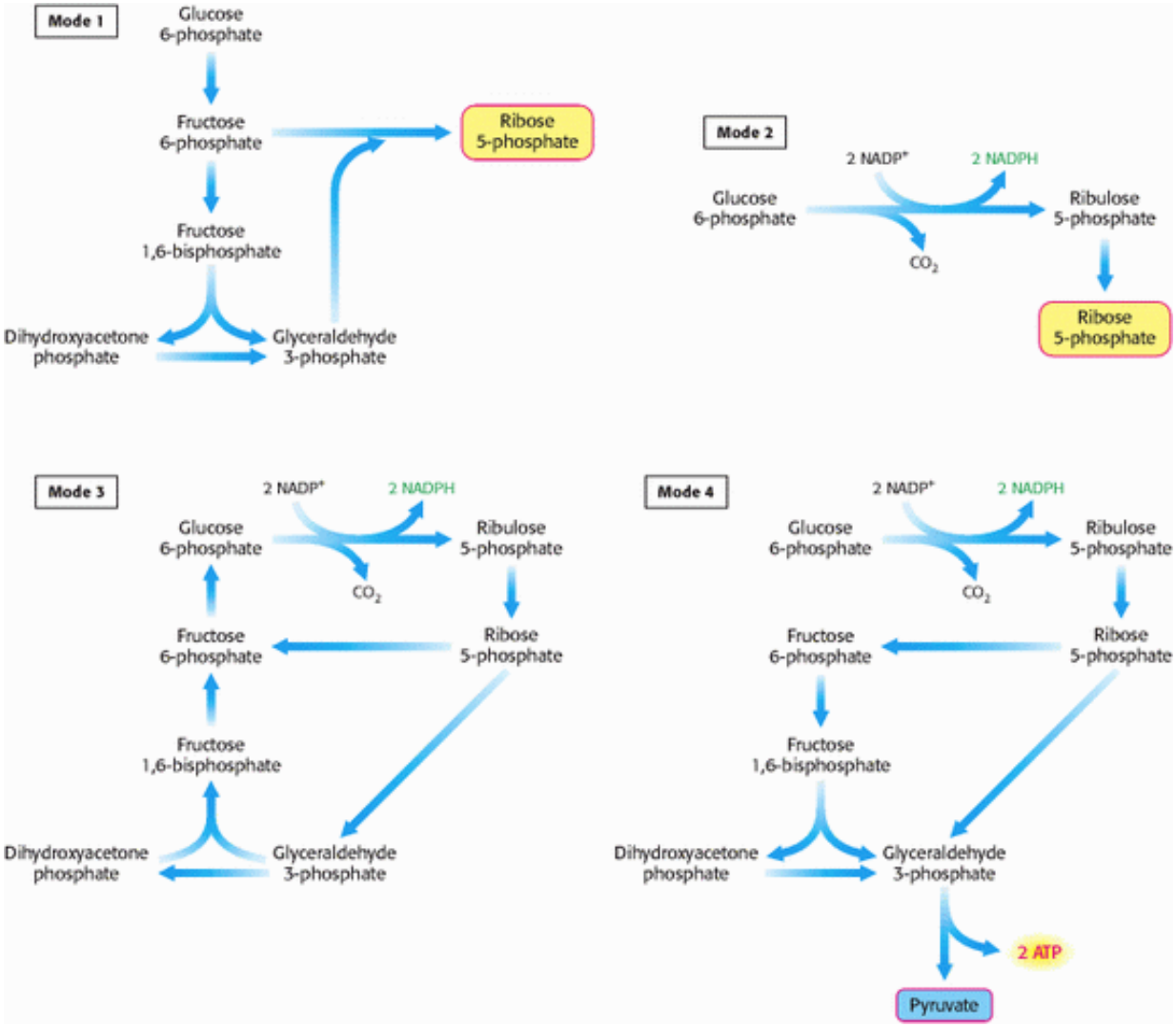
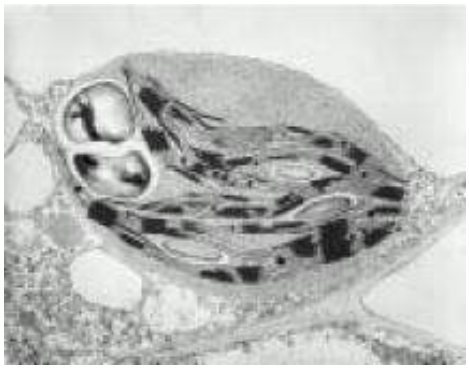


Figure 20.24. Four Modes of the Pentose Phosphate Pathway. Major products are shown in color.

Table 20.4. Tissues with active pentose phosphate pathways

Tissue	Function
Adrenal gland	Steroid synthesis
Liver	Fatty acid and cholesterol synthesis
Testes	Steroid synthesis
Adipose tissue	Fatty acid synthesis
Ovary	Steroid synthesis

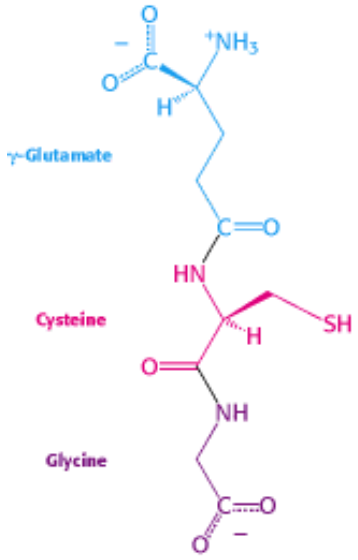
Mammary gland	Fatty acid synthesis
Red blood cells	Maintenance of reduced glutathione



Electron micrograph of a chloroplast. The thylakoid membranes course throughout the stroma of a chloroplast from a cell of *Phleum pratense*, a grass. The dark areas of stacked thylakoid membrane are grana. Several large starch granules, which store the newly synthesized glucose, are also obvious. [Biophoto Associates/Photo Researchers.]

20.5. Glucose 6-Phosphate Dehydrogenase Plays a Key Role in Protection Against Reactive Oxygen Species

Reactive oxygen species (ROS) generated in oxidative metabolism inflict damage on all classes of macromolecules and can ultimately lead to cell death. Indeed, ROS are implicated in a number of human diseases (Section 18.3.6). Reduced *glutathione* (GSH), a tripeptide with a free sulfhydryl group, is required to combat oxidative stress and maintain the normal reduced state in the cell. Oxidized glutathione (GSSG) is reduced by NADPH generated by glucose 6-phosphate dehydrogenase in the pentose phosphate pathway. Indeed, cells with reduced levels of glucose 6-phosphate dehydrogenase are especially sensitive to oxidative stress. This stress is most acute in red blood cells because, lacking mitochondria, they have no alternative means of generating reducing power.



Glutathione (reduced)
(γ-Glutamylcysteinylglycine)

20.5.1. Glucose 6-phosphate Dehydrogenase Deficiency Causes a Drug-Induced

Hemolytic Anemia

At its introduction in 1926, an antimalarial drug, pamaquine, was associated with the appearance of severe and mysterious ailments. Most patients tolerated the drug well, but a few developed severe symptoms within a few days after therapy was started. The urine turned black, jaundice developed, and the hemoglobin content of the blood dropped sharply. In some cases, massive destruction of red blood cells caused death.

This drug-induced hemolytic anemia was shown 30 years later to be caused by a *deficiency of glucose 6-phosphate dehydrogenase*, the enzyme catalyzing the first step in the oxidative branch of the pentose phosphate pathway. This defect, which is inherited on the X chromosome, is the most common enzymopathy, affecting hundreds of millions of people. The major role of NADPH in red cells is to reduce the disulfide form of glutathione to the sulfhydryl form. The enzyme that catalyzes the regeneration of reduced glutathione, the flavoprotein *glutathione reductase*, a dimer of 50-kd subunits, is homologous to ferredoxin-$NADP^+$ reductase, which we encountered in photosynthesis (Section 19.3.4). The reduced form of glutathione serves as a *sulfhydryl buffer* that maintains the cysteine residues of hemoglobin and other red-blood-cell proteins in the reduced state. Normally, the ratio of the reduced to oxidized forms of glutathione in red blood cells is 500.

How is GSH regenerated from GSSG and NADPH by glutathione reductase? The electrons from NADPH are not directly transferred to the disulfide bond in oxidized glutathione. Rather, they are transferred from NADPH to a tightly bound flavin adenine dinucleotide (FAD) on the reductase, then to a disulfide bridge between two cysteine residues in the enzyme subunit, and finally to oxidized glutathione.

Reduced glutathione is essential for maintaining the normal structure of red blood cells and for keeping hemoglobin in the ferrous state. The reduced form also plays a role in detoxification by reacting with hydrogen peroxide and organic peroxides.

$$2\,GSH + ROOH \longrightarrow GSSG + H_2O + ROH$$

Cells with a lowered level of reduced glutathione are more susceptible to hemolysis. How can we explain this phenomenon biochemically? The presence of pamaquine, a purine glycoside of fava beans, or other nonenzymatic oxidative agents leads to the generation of peroxides, reactive oxygen species that can damage membranes as well as other biomolecules. Peroxides are normally eliminated by glutathione peroxidase with the use of glutathione as a reducing agent (Section 24.4). Moreover, in the absence of the enzyme, the hemoglobin sulfhydryl groups can no longer be maintained in the reduced form and hemoglobin molecules then cross-link with one another to form aggregates called *Heinz bodies* on cell membranes (Figure 20.25). Membranes damaged by the Heinz bodies and reactive oxygen species become deformed and the cell is likely to undergo lysis. In the absence of oxidative stress, however, the deficiency is quite benign. The occurrence of this dehydrogenase deficiency also clearly demonstrates that *atypical reactions to drugs may have a genetic basis*.

20.5.2. A Deficiency of Glucose 6-phosphate Dehydrogenase Confers an Evolutionary Advantage in Some Circumstances

The incidence of the most common form of glucose 6-phosphate dehydrogenase deficiency, characterized by a tenfold reduction in enzymatic activity in red blood cells, is 11% among Americans of African heritage. This high frequency suggests that the deficiency may be advantageous under certain environmental conditions. Indeed, *glucose 6-phosphate dehydrogenase deficiency protects against falciparum malaria*. The parasites causing this disease require reduced glutathione and the products of the pentose phosphate pathway for optimal growth. Thus, glucose 6-phosphate dehydrogenase deficiency is a mechanism of protection against malaria, which accounts for its high frequency in malaria-infested regions of the world. We see here once again the interplay of heredity and environment in the production of disease.

Vicia faba. The Mediterranean plant *Vicia faba* is a source of fava beans that contain the purine glycoside vicine. [Inga Spence/ Visuals Unlimited.]

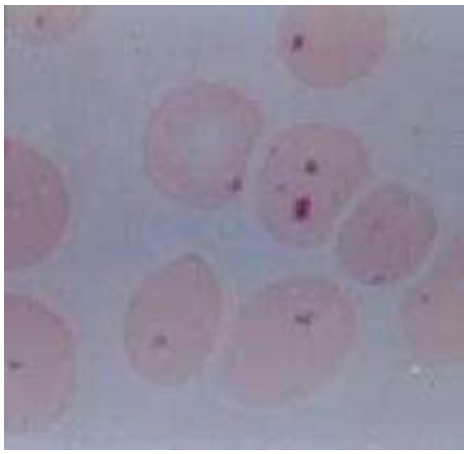


Figure 20.25. Red Blood Cells with Heinz Bodies. The light micrograph shows red blood cells obtained from a person deficient in glucose 6-phosphate dehydrogenase. The dark particles, called Heinz bodies, inside the cells are clumps of denatured protein that adhere to the plasma membrane and stain with basic dyes. Red blood cells in such people are highly susceptible to oxidative damage. [Courtesy of Dr. Stanley Schrier.]

Summary

The Calvin Cycle Synthesizes Hexoses from Carbon Dioxide and Water

ATP and NADPH formed in the light reactions of photosynthesis are used to convert CO_2 into hexoses and other organic compounds. The dark phase of photosynthesis, called the Calvin cycle, starts with the reaction of CO_2 and ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate. The steps in the conversion of 3-phosphoglycerate into fructose 6-phosphate and glucose 6-phosphate are like those of gluconeogenesis, except that glyceraldehyde 3-phosphate dehydrogenase in chloroplasts is specific for NADPH rather than NADH. Ribulose 1,5-bisphosphate is regenerated from fructose 6-phosphate, glyceraldehyde 3-phosphate, and dihydroxyacetone phosphate by a complex series of reactions. Several of the steps in the regeneration of ribulose 1,5-bisphosphate are like those of the pentose phosphate pathway. Three molecules of ATP and two molecules of NADPH are consumed for each molecule of CO_2 converted into a hexose. Starch in chloroplasts and sucrose in the cytosol are the major carbohydrate stores in plants.

The Activity of the Calvin Cycle Depends on Environmental Conditions

Reduced thioredoxin formed by the light-driven transfer of electrons from ferredoxin activates enzymes of the Calvin cycle by reducing disulfide bridges. The light-induced increase in pH and Mg^{2+} level of the stroma is important in stimulating the carboxylation of ribulose 1,5-bisphosphate by ribulose 1,5-bisphosphate carboxylase. This enzyme also catalyzes a competing oxygenase reaction, which produces phosphoglycolate and 3-phosphoglycerate. The recycling of phosphoglycolate leads to the release of CO_2 and further consumption of O_2 in a process called photorespiration. This wasteful side reaction is minimized in tropical plants, which have an accessory pathway—called the C_4 pathway—for concentrating CO_2 at the site of the Calvin cycle. This pathway enables tropical plants to take advantage of high levels of light and minimize the oxygenation of ribulose 1,5-bisphosphate. Plants in arid ecosystems employ Crassulacean acid metabolism (CAM) to prevent dehydration. In CAM plants, the C_4 pathway is active during the night when the plant exchanges gases with the air. During the day, gas exchange is eliminated and CO_2 is generated from malate stored in vacuoles.

The Pentose Phosphate Pathway Generates NADPH and Synthesizes Five-Carbon Sugars

Whereas the Calvin cycle is present only in photosynthetic organisms, the pentose phosphate pathway is present in all organisms. The pentose phosphate pathway generates NADPH and ribose 5-phosphate in the cytosol. NADPH is used in reductive biosyntheses, whereas ribose 5-phosphate is used in the synthesis of RNA, DNA, and nucleotide coenzymes. The pentose phosphate pathway starts with the dehydrogenation of glucose 6-phosphate to form a lactone, which is hydrolyzed to give 6-phosphogluconate and then oxidatively decarboxylated to yield ribulose 5-phosphate. $NADP^+$ is the electron acceptor in both of these oxidations. The last step is the isomerization of ribulose 5-phosphate (a ketose) to ribose 5-phosphate (an aldose). A different mode of the pathway is active when cells need much more NADPH than ribose 5- phosphate. Under these conditions, ribose 5-phosphate is converted into glyceraldehyde 3-phosphate and fructose 6-phosphate by transketolase and transaldolase. These two enzymes create a reversible link between the pentose phosphate pathway and glycolysis. Xylulose 5-phosphate, sedoheptulose 7-phosphate, and erythrose 4-phosphate are intermediates in these interconversions. In this way, 12 molecules of NADPH can be generated for each molecule of glucose 6-phosphate that is completely oxidized to CO_2.

The Metabolism of Glucose 6-phosphate by the Pentose Phosphate Pathway Is Coordinated with Glycolysis

Only the nonoxidative branch of the pathway is significantly active when much more ribose 5-phosphate than NADPH needs to be synthesized. Under these conditions, fructose 6-phosphate and glyceraldehyde 3-phosphate (formed by the glycolytic pathway) are converted into ribose 5-phosphate without the formation of NADPH. Alternatively, ribose 5-phosphate formed by the oxidative branch can be converted into pyruvate through fructose 6-phosphate and glyceraldehyde 3-phosphate. In this mode, ATP and NADPH are generated, and five of the six carbons of glucose 6-phosphate emerge in pyruvate. The interplay of the glycolytic and pentose phosphate pathways enables the levels of NADPH, ATP, and building blocks such as ribose 5-phosphate and pyruvate to be continuously adjusted to meet cellular needs.

Glucose 6-phosphate Dehydrogenase Plays a Key Role in Protection Against Reactive Oxygen Species

NADPH generated by glucose 6-phosphate dehydrogenase maintains the appropriate levels of reduced glutathione required to combat oxidative stress and maintain the proper reducing environment in the cell. Cells with diminished glucose 6-phosphate dehydrogenase activity are especially sensitive to oxidative stress.

Key Terms

Calvin cycle (dark reactions)

autotroph

heterotroph

rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase)

peroxisome

photorespiration

starch

sucrose

thioredoxin

C_4 pathway

C_4 plant

C_3 plant

Crassulacean acid metabolism (CAM)

pentose phosphate pathway

glucose 6-phosphate dehydrogenase

transketolase

transaldolase

glutathione

Problems

1. *Variation on a theme.* Sedoheptulose 1,7-bisphosphate is an intermediate in the Calvin cycle but not in the pentose phosphate pathway. What is the enzymatic basis of this difference?

   See answer

2. *Total eclipse*. An illuminated suspension of *Chlorella* is actively carrying out photosynthesis. Suppose that the light is suddenly switched off. How would the levels of 3-phosphoglycerate and ribulose 1,5-bisphosphate change in the next minute?

   See answer

3. *CO_2 deprivation.* An illuminated suspension of *Chlorella* is actively carrying out photosynthesis in the presence of 1% CO_2. The concentration of CO_2 is abruptly reduced to 0.003%. What effect would this reduction have on the levels of 3-phosphoglycerate and ribulose 1,5-bisphosphate in the next minute?

   See answer

4. *A potent analog.* 2-Carboxyarabinitol 1,5-bisphosphate (CABP) has been useful in studies of rubisco.

   (a) Write the structural formula of CABP.

   (b) Which catalytic intermediate does it resemble?

   (c) Predict the effect of CABP on rubisco.

   See answer

5. *Salvage operation.* Write a balanced equation for the transamination of glyoxylate to yield glycine.

   See answer

6. *When one equals two.* In the C_4 pathway, one ATP molecule is used in combining the CO_2 with phosphoenolpyruvate to form oxaloacetate (Figure 20.17), but, in the computation of energetics bookkeeping, two ATP molecules are said to be consumed. Explain.

   See answer

7. *Dog days of August.* Before the days of pampered lawns, most homeowners practiced horticultural Darwinism. A result was that the lush lawns of early summer would often convert into robust cultures of crabgrass in the dog days of August. Provide a possible biochemical explanation for this transition.

   See answer

8. *Global warming.* C_3 plants are most common in higher latitudes and become less common at latitudes near the equator. The reverse is true of C_4 plants. How might global warming affect this distribution?

See answer

9. *Tracing glucose.* Glucose labeled with ^{14}C at C-6 is added to a solution containing the enzymes and cofactors of the oxidative phase of the pentose phosphate pathway. What is the fate of the radioactive label?

See answer

10. *Recurring decarboxylations.* Which reaction in the citric acid cycle is most analogous to the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate? What kind of enzyme-bound intermediate is formed in both reactions?

See answer

11. *Carbon shuffling.* Ribose 5-phosphate labeled with ^{14}C at C-1 is added to a solution containing transketolase, transaldolase, phosphopentose epimerase, phosphopentose isomerase, and glyceraldehyde 3-phosphate. What is the distribution of the radioactive label in the erythrose 4-phosphate and fructose 6-phosphate that are formed in this reaction mixture?

See answer

12. *Synthetic stoichiometries.* What is the stoichiometry of synthesis of (a) ribose 5-phosphate from glucose 6-phosphate without the concomitant generation of NADPH; (b) NADPH from glucose 6-phosphate without the concomitant formation of pentose sugars?

See answer

13. *Trapping a reactive lysine.* Design a chemical experiment to identify the lysine residue that forms a Schiff base at the active site of transaldolase.

See answer

14. *Reductive power.* What ratio of NADPH to $NADP^+$ is required to sustain [GSH] = 10 mM and [GSSG] = 1 mM? Use the redox potentials given in Table 18.1.

See answer

Mechanism Problems

15. *An alternative approach.* The mechanisms of some aldolases do not include Schiff-base intermediates. Instead, these enzymes require bound metal ions. Propose such a mechanism for the conversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate into fructose 1,6-bisphosphate.

See answer

16. *A recurring intermediate.* Phosphopentose isomerase interconverts the aldose ribose 5-phosphate and the ketose ribulose 5-phosphate. Propose a mechanism.

See answer

Chapter Integration Problems

17. *Catching carbons.* Radioactive-labeling experiments can yield estimates of how much glucose 6-phosphate is metabolized by the pentose phosphate pathway and how much is metabolized by the combined action of glycolysis and the citric acid cycle. Suppose that you have samples of two different tissues as well as two radioactively labeled glucose samples, one with glucose labeled with ^{14}C at C-1 and the other with glucose labeled with ^{14}C at C-6. Design an experiment that would enable you to determine the relative activity of the aerobic metabolism of glucose compared with metabolism by the pentose phosphate pathway.

See answer

18. *Photosynthetic efficiency.* Use the following information to estimate the efficiency of photosynthesis.

The $\Delta G^{\circ\prime}$ for the reduction of CO_2 to the level of hexose is +114 kcal mol^{-1} (+477 kJ mol^{-1}).

A mole of 600-nm photons has an energy content of 47.6 kcal (199 kJ).

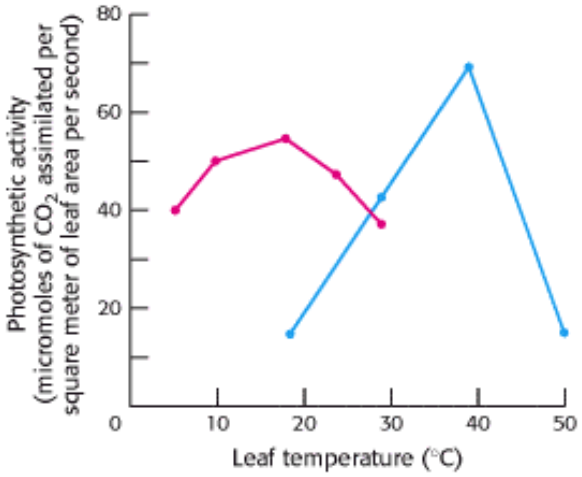
Assume that the proton gradient generated in producing the required NADPH is sufficient to drive the synthesis of the required ATP.

See answer

Date Interpretation Problem

19. Graph A shows the photosynthetic activity of two species of plant, one a C_4 plant and the other a C_3 plant, as a function of leaf temperature.

(A)

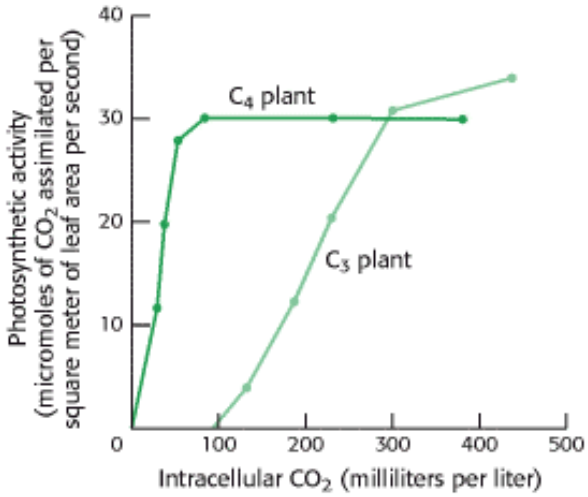


(a) Which data were most likely generated by the C_4 plant and which by the C_3 plant? Explain.

(b) Suggest some possible explanations for why the photosynthetic activity falls at higher temperatures.

Graph B illustrates how the photosynthetic activity of C_3 and C_4 plants varies with CO_2 concentration when temperature (30°C) and light intensity (high) are constant.

(B)



(c) Why can C_4 plants thrive at CO_2 concentrations that do not support the growth of C_3 plants?

(d) Suggest a plausible explanation for why C_3 plants continue to increase photosynthetic activity at higher CO_2 concentrations, whereas C_4 plants reach a plateau.

See answer

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21. Glycogen Metabolism

Glycogen is a *readily mobilized storage form of glucose.* It is a very large, branched polymer of glucose residues (Figure 21.1) that can be broken down to yield glucose molecules when energy is needed. Most of the glucose residues in glycogen are linked by α-1,4-glycosidic bonds. Branches at about every tenth residue are created by α-1,6-glycosidic bonds. Recall that α-glycosidic linkages form open helical polymers, whereas β linkages produce nearly straight strands that form structural fibrils, as in cellulose (Section 11.2.3).

Glycogen is not as reduced as fatty acids are and consequently not as energy rich. Why do animals store any energy as glycogen? Why not convert all excess fuel into fatty acids? Glycogen is an important fuel reserve for several reasons. The controlled breakdown of glycogen and release of glucose increase the amount of glucose that is available between meals. Hence, glycogen serves as a buffer to maintain blood-glucose levels. Glycogen's role in maintaining blood-glucose levels is especially important because glucose is virtually the only fuel used by the brain, except during prolonged starvation. Moreover, the glucose from glycogen is readily mobilized and is therefore a good source of energy for sudden, strenuous activity. Unlike fatty acids, the released glucose can provide energy in the absence of oxygen and can thus supply energy for anaerobic activity.

The two major sites of glycogen storage are the liver and skeletal muscle. The concentration of glycogen is higher in the liver than in muscle (10% versus 2% by weight), but more glycogen is stored in skeletal muscle overall because of its much greater mass. Glycogen is present in the cytosol in the form of granules ranging in diameter from 10 to 40 nm (Figure 21.2). In the liver, glycogen synthesis and degradation are regulated to maintain blood-glucose levels as required to meet the needs of the organism as a whole. In contrast, in muscle, these processes are regulated to meet the energy needs of the muscle itself.

21.0.1. An Overview of Glycogen Metabolism

Glycogen degradation and synthesis are relatively simple biochemical processes. Glycogen degradation consists of three steps: (1) the release of glucose 1-phosphate from glycogen, (2) the remodeling of the glycogen substrate to permit further degradation, and (3) the conversion of glucose 1-phosphate into glucose 6-phosphate for further metabolism. The glucose 6-phosphate derived from the breakdown of glycogen has three fates (Figure 21.3): (1) It is the initial substrate for glycolysis, (2) it can be processed by the pentose phosphate pathway to yield NADPH and ribose derivatives; and (3) it can be converted into free glucose for release into the bloodstream. This conversion takes place mainly in the liver and to a lesser extent in the intestines and kidneys.

Glycogen synthesis requires an activated form of glucose, uridine diphosphate glucose (UDP-glucose), which is formed by the reaction of UTP and glucose 1-phosphate. UDP-glucose is added to the nonreducing end of glycogen molecules. As is the case for glycogen degradation, the glycogen molecule must be remodeled for continued synthesis.

The regulation of these processes is quite complex. Several enzymes taking part in glycogen metabolism allosterically respond to metabolites that signal the energy needs of the cell. *These allosteric responses allow the adjustment of enzyme activity to meet the needs of the cell in which the enzymes are expressed.* Glycogen metabolism is also regulated by hormonally stimulated cascades that lead to the reversible phosphorylation of enzymes, which alters their kinetic properties. *Regulation by hormones allows glygogen metabolism to adjust to the needs of the entire organism.* By both these mechanisms, glycogen degradation is integrated with glycogen synthesis. We will first examine the metabolism, followed by enzyme regulation and then the elaborate integration of control mechanisms.

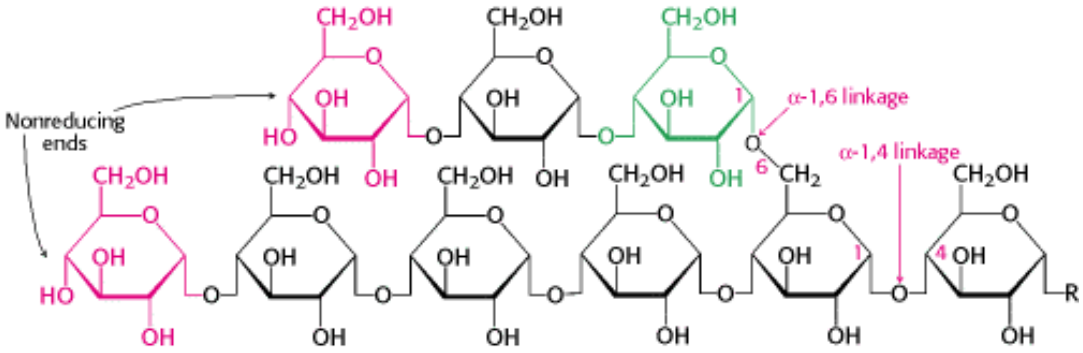


Figure 21.1. Glycogen Structure. In this structure of two outer branches of a glycogen molecule, the residues at the nonreducing ends are shown in red and residue that starts a branch is shown in green. The rest of the glycogen molecule is represented by R.

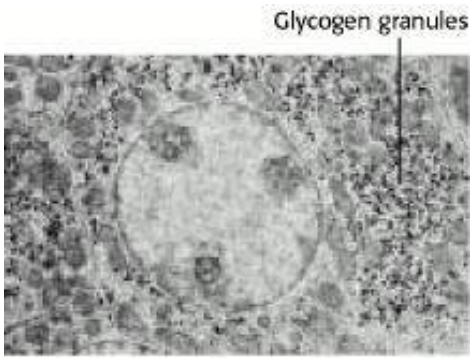


Figure 21.2. Electron Micrograph of a Liver Cell. The dense particles in the cytoplasm are glycogen granules. [Courtesy of Dr. George Palade.]

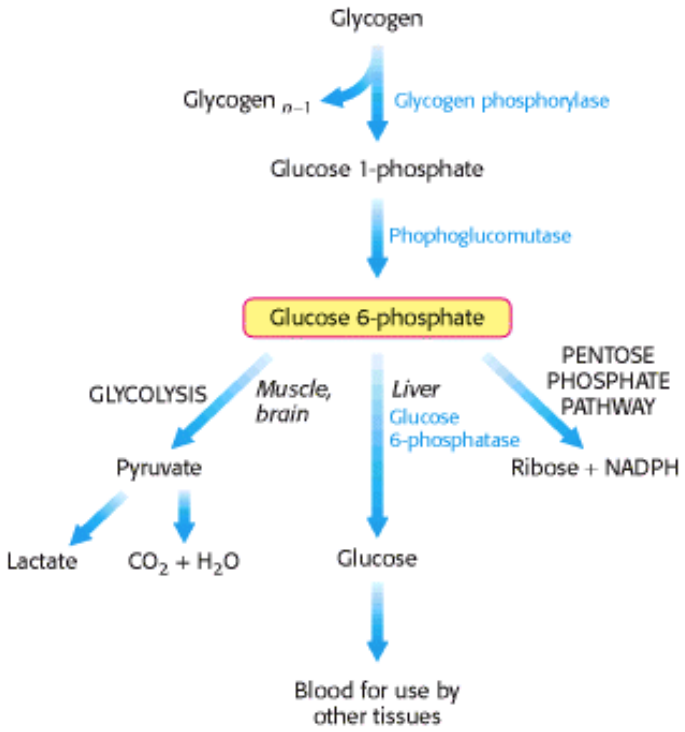
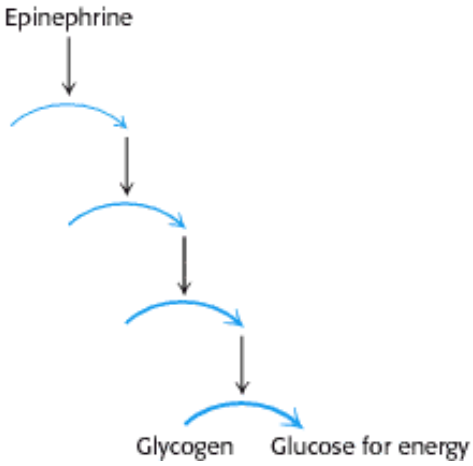


Figure 21.3. Fates of Glucose 6-Phosphate. Glucose 6-phosphate derived from glycogen can (1) be used as a fuel for

anaerobic or aerobic metabolism as in, for instance, muscle; (2) be converted into free glucose in the liver and subsequently released into the blood; (3) be processed by the pentose phosphate pathway to generate NADPH or ribose in a variety of tissues.



Signal cascades lead to the mobilization of glycogen to produce glucose, an energy source for runners. [(Left) Mike Powell/Allsport.]

21.1. Glycogen Breakdown Requires the Interplay of Several Enzymes

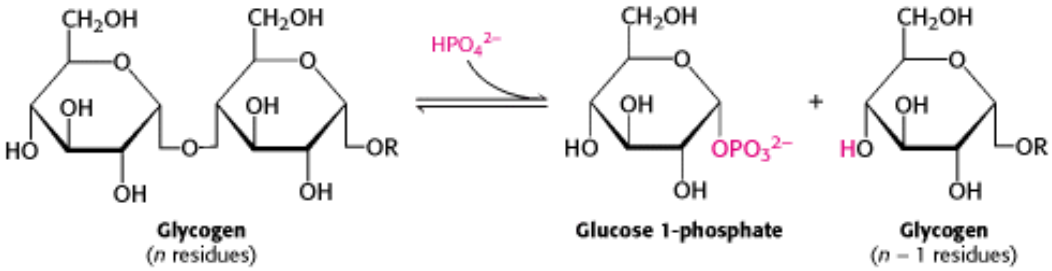
The efficient breakdown of glycogen to provide glucose 6-phosphate for further metabolism requires four enzyme activities: one to degrade glycogen, two to remodel glycogen so that it remains a substrate for degradation, and one to convert the product of glycogen breakdown into a form suitable for further metabolism. We will examine each of these activities in turn.

21.1.1. Phosphorylase Catalyzes the Phosphorolytic Cleavage of Glycogen to Release Glucose 1-phosphate

Glycogen phosphorylase, the key enzyme in glycogen breakdown, cleaves its substrate by the addition of orthophosphate (P_i) to yield *glucose 1-phosphate*. The cleavage of a bond by the addition of orthophosphate is referred to as *phosphorolysis*.

$$\underset{(n\ \text{residues})}{\text{Glycogen}} + P_i \rightleftharpoons \text{glucose 1-phosphate} + \underset{(n-1\ \text{residues})}{\text{glycogen}}$$

Phosphorylase catalyzes the sequential removal of glycosyl residues from the nonreducing ends of the glycogen molecule (the ends with a free 4-OH groups; Section 11.1.3). Orthophosphate splits the glycosidic linkage between C-1 of the terminal residue and C-4 of the adjacent one. Specifically, it cleaves the bond between the C-1 carbon atom and the glycosidic oxygen atom, and the α configuration at C-1 is retained.



Glucose 1-phosphate released from glycogen can be readily converted into glucose 6-phosphate (Section 21.1.3), an important metabolic intermediate, by the enzyme phosphoglucomutase.

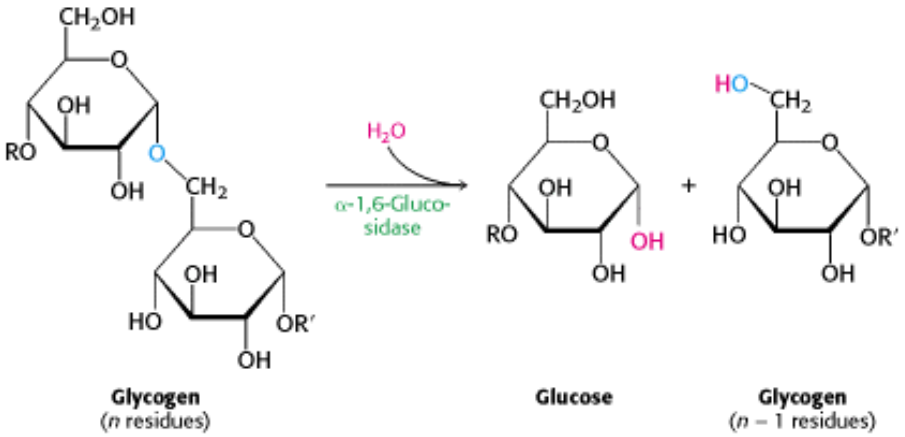
The reaction catalyzed by phosphorylase is readily reversible in vitro. At pH 6.8, the equilibrium ratio of orthophosphate to glucose 1-phosphate is 3.6. The value of $\Delta G^{\circ\prime}$ for this reaction is small because a glycosidic bond is replaced by a phosphoryl ester bond that has a nearly equal transfer potential. However, phosphorolysis proceeds far in the direction of glycogen breakdown in vivo because the $[P_i]/[\text{glucose 1-phosphate}]$ ratio is usually greater than 100, substantially favoring phosphorolysis. We see here an example of how the cell can alter the free-energy change of a reaction to favor the reaction's occurrence by altering the ratio of substrate and product.

The phosphorolytic cleavage of glycogen is energetically advantageous because the released sugar is already phosphorylated. In contrast, a hydrolytic cleavage would yield glucose, which would then have to be phosphorylated at the expense of the hydrolysis of a molecule of ATP to enter the glycolytic pathway. An additional advantage of phosphorolytic cleavage for muscle cells is that glucose 1-phosphate, negatively charged under physiological conditions, cannot diffuse out of the cell.

21.1.2. A Debranching Enzyme Also Is Needed for the Breakdown of Glycogen

Glycogen phosphorylase, the key enzyme in glycogen breakdown, can carry out this process by itself only to a limited extent before encountering an obstacle. The α-1,6-glycosidic bonds at the branch points are not susceptible to cleavage by phosphorylase. Indeed, phosphorylase stops cleaving α-1,4 linkages when it reaches a terminal residue four residues away from a branch point. Because about 1 in 10 residues is branched, glycogen degradation by the phosphorylase alone would come to a halt after the release of six glucose molecules per branch.

How can the remainder of the glycogen molecule be mobilized for use as a fuel? Two additional enzymes, a *transferase* and α-*1,6-glucosidase*, remodel the glycogen for continued degradation by the phosphorylase (Figure 21.4). *The transferase shifts a block of three glycosyl residues from one outer branch to the other.* This transfer exposes a single glucose residue joined by an α-1,6-glycosidic linkage. α-1,6-Glucosidase, also known as the debranching enzyme, hydrolyzes the α-1, 6-glycosidic bond, resulting in the release of a free glucose molecule.



This free glucose molecule is phosphorylated by the glycolytic enzyme hexokinase. Thus, the transferase and α-1,6-glucosidase convert the branched structure into a linear one, which paves the way for further cleavage by phosphorylase. It is noteworthy that, in eukaryotes, the transferase and the α-1,6-glucosidase activities are present in a single 160-kd polypeptide chain, providing yet another example of a bifunctional enzyme (Section 16.2.2). Furthermore, these enzymes may have additional features in common (Section 21.4.3).

21.1.3. Phosphoglucomutase Converts Glucose 1-phosphate into Glucose 6-phosphate

Glucose 1-phosphate formed in the phosphorolytic cleavage of glycogen must be converted into glucose 6-phosphate to enter the metabolic mainstream. This shift of a phosphoryl group is catalyzed by *phosphoglucomutase.* Recall that this enzyme is also used in galactose metabolism (Section 16.1.11). To effect this shift, the enzyme engages in the exchange of a phosphoryl group with the substrate (Figure 21.5).

The catalytic site of an active mutase molecule contains a phosphorylated serine residue. The phosphoryl group is transferred from the serine residue to the C-6 hydroxyl group of glucose 1-phosphate to form glucose 1,6-bisphosphate. The C-1 phosphoryl group of this intermediate is then shuttled to the same serine residue, resulting in the formation of glucose 6-phosphate and the regeneration of the phosphoenzyme.

These reactions are like those of *phosphoglycerate mutase*, a glycolytic enzyme (Section 16.1.7). The role of glucose 1,6-bisphosphate in the interconversion of the phosphoglucoses is like that of 2,3-bisphosphoglycerate (2,3-BPG) in the interconversion of 2-phosphoglycerate and 3-phosphoglycerate in glycolysis. A phosphoenzyme intermediate participates in both reactions.

21.1.4. Liver Contains Glucose 6-phosphatase, a Hydrolytic Enzyme Absent from Muscle

A major function of the liver is to maintain a near constant level of glucose in the blood. The liver releases glucose into the blood during muscular activity and between meals to be taken up primarily by the brain and skeletal muscle. However, the phosphorylated glucose produced by glycogen breakdown, in contrast with glucose, is not readily transported out of cells. The liver contains a hydrolytic enzyme, *glucose 6-phosphatase*, which cleaves the phosphoryl group to form free glucose and orthophosphate. This glucose 6-phosphatase, located on the lumenal side of the smooth endoplasmic reticulum membrane, is the same enzyme that releases free glucose at the conclusion of gluconeogenesis. Recall that glucose 6-phosphate is transported into the endoplasmic reticulum; glucose and orthophosphate formed by hydrolysis are then shuttled back into the cytosol (Section 16.3.5).

$$\text{Glucose 6-phosphate} + H_2O \longrightarrow \text{glucose} + P_i$$

Glucose 6-phosphatase is absent from most other tissues. Consequently, glucose 6-phosphate is retained for the

generation of ATP. In contrast, glucose is not a major fuel for the liver.

21.1.5. Pyridoxal Phosphate Participates in the Phosphorolytic Cleavage of Glycogen

Let us now examine the catalytic mechanism of glycogen phosphorylase, which is a dimer of two identical 97-kd subunits. Each subunit is compactly folded into an *amino-terminal domain* (480 residues) containing a *glycogen-binding site* and a *carboxyl-terminal domain* (360 residues; Figure 21.6). The catalytic site is located in a deep crevice formed by residues from amino- and carboxyl-terminal domains. The special challenge faced by phosphorylase is to cleave glycogen phosphorolytically rather than hydrolytically to save the ATP required to phosphorylate free glucose. This cleavage requires that water be excluded from the active site. Several clues provide us with information about the mechanism by which phosphorylase achieves the exclusion of water. First, both the glycogen substrate and the glucose 1-phosphate product have an α configuration at C-1 (the designation α means that the oxygen atom attached to C-1 is below the plane of the ring; Section 11.1.3). A direct attack of phosphate on C-1 of a sugar would invert the configuration at this carbon because the reaction would proceed through a pentacovalent transition state. Because the resulting glucose 1-phosphate has an α rather than a β configuration, an even number of steps (most simply, two) is required. The most likely explanation for these results is that a *carbonium ion intermediate* is formed.

A second clue to the catalytic mechanism of phosphorylase is its requirement for *pyridoxal phosphate (PLP),* a derivative of pyridoxine (vitamin B_6, Section 8.6.1). The aldehyde group of this coenzyme forms a Schiff base with a specific lysine side chain of the enzyme (Figure 21.7). The results of structural studies indicate that the reacting orthophosphate group takes a position between the 5′-phosphate group of PLP and the glycogen substrate (Figure 21.8). *The 5′-phosphate group of PLP acts in tandem with orthophosphate by serving as a proton donor and then as a proton acceptor (that is, as a general acid-base catalyst).* Orthophosphate (in the HPO_4^{2-} form) donates a proton to the oxygen atom attached to carbon 4 of the departing glycogen chain and simultaneously acquires a proton from PLP. The carbonium ion intermediate formed in this step is then attacked by orthophosphate to form α-glucose 1-phosphate, with the concomitant return of a hydrogen atom to pyridoxal phosphate. The requirement that water be excluded from the active site calls for the special role of pyridoxal phosphate in facilitating the phosphorolytic cleavage.

The glycogen-binding site is 30 Å away from the catalytic site (see Figure 21.6), but it is connected to the catalytic site by a narrow crevice able to accommodate four or five glucose units. The large separation between the binding site and the catalytic site enables the enzyme to phosphorolyze many residues without having to dissociate and reassociate after each catalytic cycle. An enzyme that can catalyze many reactions without having to dissociate and reassociate after each catalytic step is said to be *processive* —a property of enzymes that synthesize and degrade large polymers. We will see such enzymes again when we consider DNA and RNA synthesis.

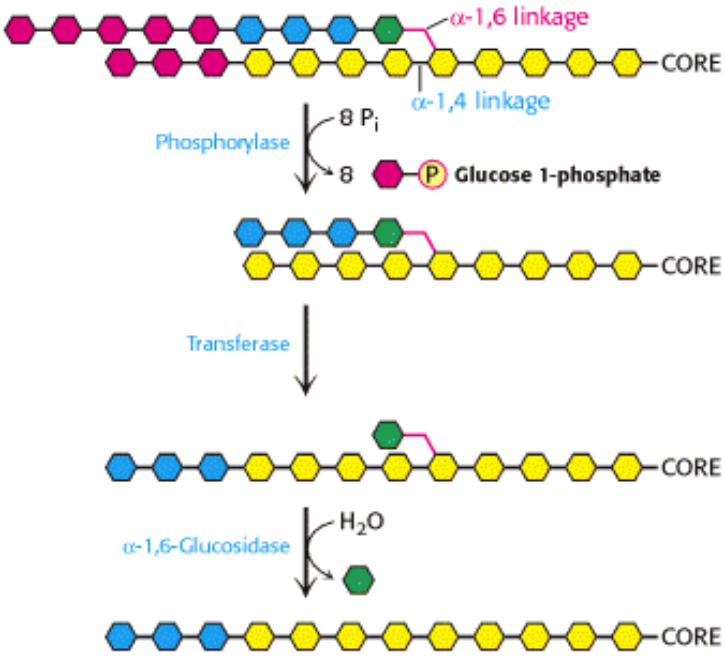


Figure 21.4. Glycogen Remodeling. First, α-1,4-glycosidic bonds on each branch are cleaved by phosphorylase, leaving four residues along each branch. The transferase shifts a block of three glycosyl residues from one outer branch to the other. In this reaction, the α-1,4-glycosidic link between the blue and the green residues is broken and a new α-1,4 link between the blue and the yellow residues is formed. The green residue is then removed by α-1,6-glucosidase, leaving a linear chain with all α-1,4 linkages, suitable for further cleavage by phosphorylase.

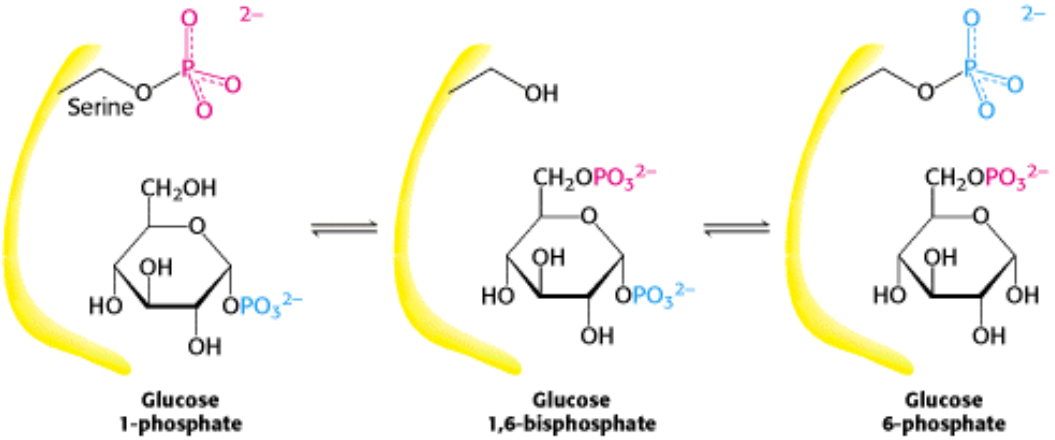


Figure 21.5. Reaction Catalyzed by Phosphoglucomutase. A phosphoryl group is transferred from the enzyme to the substrate, and a different phosphoryl group is transferred back to restore the enzyme to its initial state.

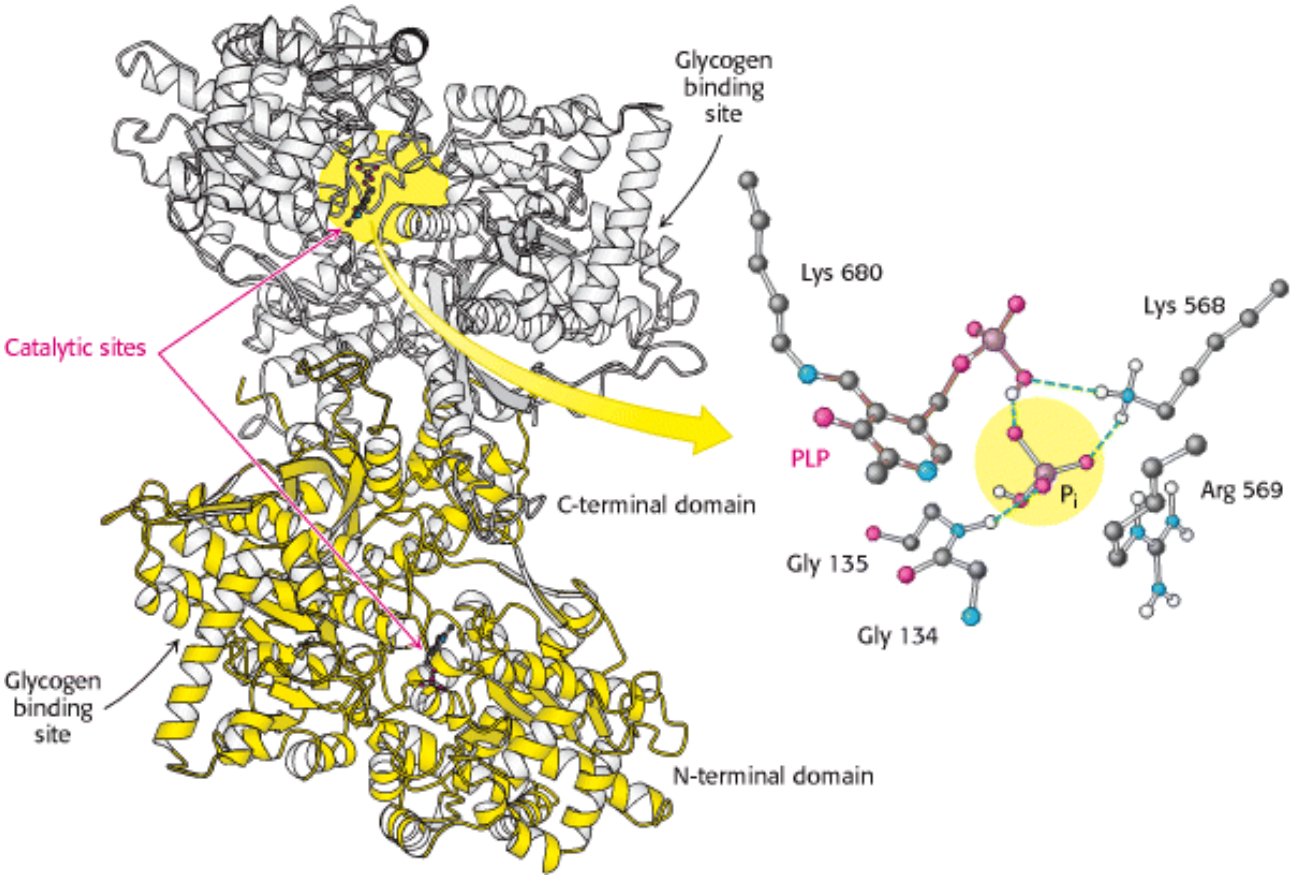


Figure 21.6. Structure of Glycogen Phosphorylase. This enzyme forms a homodimer: one subunit is shown in white and the other in yellow. Each catalytic site includes a pyridoxal-phosphate (PLP) group, linked to lysine 680 of the enzyme. The binding site for the phosphate (P_i) substrate is shown.

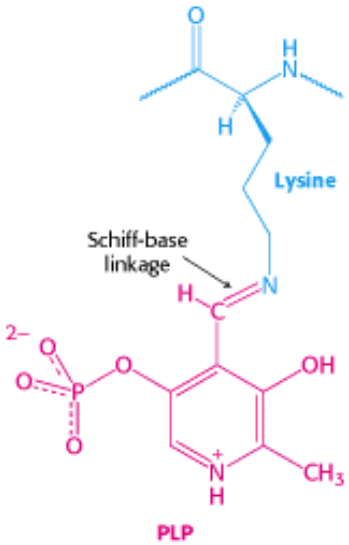


Figure 21.7. PLP-Schiff-Base Linkage. A pyridoxal phosphate group (red) forms a Schiff base with a lysine residue (blue) at the active site of phosphorylase.

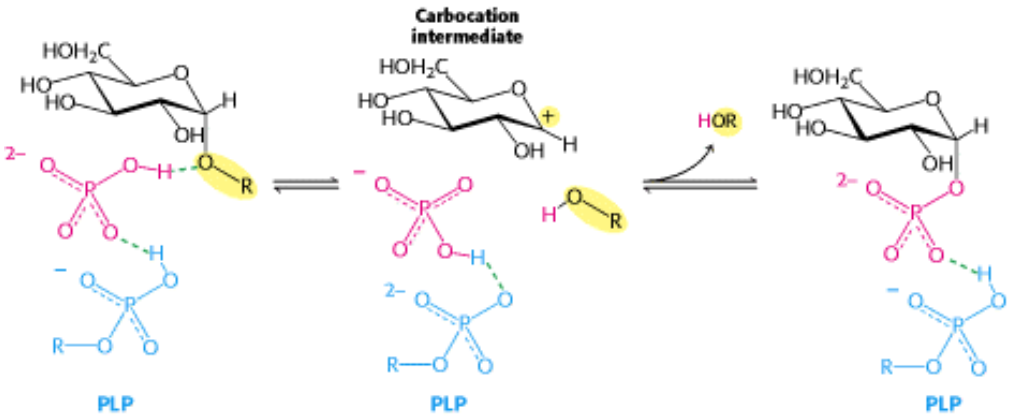


Figure 21.8. Phosphorylase Mechanism. A bound $HPO4^{2-}$ group (red) favors the cleavage of the glycosidic bond by donating a proton to the departing glucose (black). This reaction results in the formation of a carbocation and is favored by the transfer of a proton from the protonated phosphate group of the bound pyridoxal phosphate PLP group (blue). The combination of the carbocation and the orthophosphate results in the formation of glucose 1-phosphate.

21.2. Phosphorylase Is Regulated by Allosteric Interactions and Reversible Phosphorylation

Structural Insights, Glycogen Phosphorylase, looks closely at the structural mechanisms of phosphorylase regulation, examining the efects of allosteric effectors and serine phosphorylation.

Glycogen metabolism is precisely controlled by multiple interlocking mechanisms, and the focus of this control is glycogen phosphorylase. *Phosphorylase is regulated by several allosteric effectors that signal the energy state of the cell as well as by reversible phosphorylation, which is responsive to hormones such as insulin, epinephrine, and glucagon.* We will examine the differences in the control of glycogen metabolism in two tissues: skeletal muscle and liver. These differences are due to the fact that *the muscle uses glucose to produce energy for itself, whereas the liver maintains glucose homeostasis of the organism as a whole.*

21.2.1. Muscle Phosphorylase Is Regulated by the Intracellular Energy Charge

We begin by considering the glycogen phosphorylase from muscle. The dimeric skeletal muscle phosphorylase exists in two interconvertible forms: a *usually active* phosphorylase *a* and a *usually inactive* phosphorylase *b* (Figure 21.9). Each of these two forms exists in equilibrium between an active relaxed (R) state and a much less active tense (T) state, but the equilibrium for phosphorylase *a* favors the R state whereas the equilibrium for phosphorylase *b* favors the T state (Figure 21.10). Phosphorylase *a* and phosphorylase *b* differ by a single phosphoryl group in each subunit. Phosphorylase *b* is converted into phosphorylase *a* when it is phosphorylated at a single serine residue (serine 14) in each subunit. The regulatory enzyme *phosphorylase kinase* catalyzes this covalent modification. As will be described, increased levels of epinephrine (resulting from fear or from the excitement of exercise) and the electrical stimulation of muscle result in phosphorylation of the enzyme to the phosphorylase *a* form.

Comparison of the structures of phosphorylase *a* and phosphorylase *b* reveals that subtle structural changes at the subunit interfaces are transmitted to the active sites (see Figure 21.9). The transition from the T state (represented by phosphorylase *b*) to the R state (represented by phosphorylase *a*) entails a 10-degree rotation around the twofold axis of the dimer. Most importantly, this transition is associated with structural changes in α helices that move a loop out of the active site of each subunit. Thus, the T state is less active because the catalytic site is partly blocked. In the R state, the catalytic site is more accessible and a binding site for orthophosphate is well organized.

The position of the equilibrium of phosphorylase *b* between the T and the R form is responsive to conditions in the cell. Muscle phosphorylase *b* is active only in the presence of high concentrations of AMP, which binds to a nucleotide-binding site and stabilizes the conformation of phosphorylase *b* in the R state (Figure 21.11). ATP acts as a negative allosteric effector by competing with AMP and so favors the T state. Thus, *the transition of phosphorylase* b *between the T and the R state is controlled by the energy charge of the muscle cell.* Glucose 6-phosphate also favors the T state of phosphorylase *b*, an example of feedback inhibition.

Under most physiological conditions, *phosphorylase* b *is inactive because of the inhibitory effects of ATP and glucose 6-phosphate.* In contrast, *phosphorylase* a *is fully active*, regardless of the levels of AMP, ATP, and glucose 6-phosphate. In resting muscle, nearly all the enzyme is in the inactive *b* form. When exercise commences, the elevated level of AMP leads to the activation of phosphorylase *b*. Exercise will also result in hormone release that generates the phosphorylated *a* form of the enzyme. The absence of glucose 6-phosphatase in muscle ensures that glucose 6-phosphate derived from glycogen remains within the cell for energy transformation.

21.2.2. Liver Phosphorylase Produces Glucose for Use by Other Tissues

The regulation of liver glycogen phosphorylase differs markedly from that of muscle, a consequence of the role of the liver in glucose homeostasis for the organism as a whole. In human beings, liver phosphorylase and muscle phosphorylase are approximately 90% identical in amino acid sequence. The differences result in subtle but important shifts in the stability of various forms of the enzyme. In contrast with the muscle enzyme, liver phosphorylase *a* but not *b* exhibits the most responsive T-to-R transition. The binding of glucose shifts the allosteric equilibrium of the *a* form from the R to the T state, deactivating the enzyme (Figure 21.12). Why would glucose function as a negative regulator of liver phosphorylase *a*? The role of glycogen degradation in the liver is to form glucose for *export to other tissues* when the blood-glucose level is low. Hence, if free glucose is present from some other source such as diet, there is no need to mobilize glycogen. Unlike the enzyme in muscle, the liver phosphorylase is insensitive to regulation by AMP because the liver does not undergo the dramatic changes in energy charge seen in a contracting muscle. We see here a clear example of the use of isozymic forms of the same enzyme to establish the tissue-specific biochemical properties of muscle and the liver.

21.2.3. Phosphorylase Kinase Is Activated by Phosphorylation and Calcium Ions

The next upstream component of this signal-transduction pathway is the enzyme that covalently modifies phosphorylase. This enzyme is *phosphorylase kinase*, a very large protein with a subunit composition in skeletal muscle of $(\alpha\,\beta\,\gamma\,\delta)_4$ and a mass of 1200 kd. The catalytic activity resides in the γ subunit, whereas the other subunits serve a regulatory function. This kinase is under dual control. Like its own substrate, phosphorylase kinase is regulated by phosphorylation: the kinase is converted from *a low-activity form into a high-activity one by phosphorylation of its* β *subunit.* The enzyme catalyzing the activation of phosphorylase kinase is *protein kinase A (PKA)*, which is switched on by a second messenger, cyclic AMP (Sections 10.4.2 and 15.1.5). As will be discussed, hormones such as epinephrine induce the breakdown of glycogen by activating a cyclic AMP cascade (Figure 21.13).

Phosphorylase kinase can also be partly activated by Ca^{2+} levels of the order of 1 μM. Its δ subunit is *calmodulin*, a calcium sensor that stimulates many enzymes in eukaryotes (Section 15.3.2). This mode of activation of the kinase is important in muscle, where contraction is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum. Phosphorylase kinase attains maximal activity only after both phosphorylation of the β subunit and activation of the δ subunit by Ca^{2+} binding.

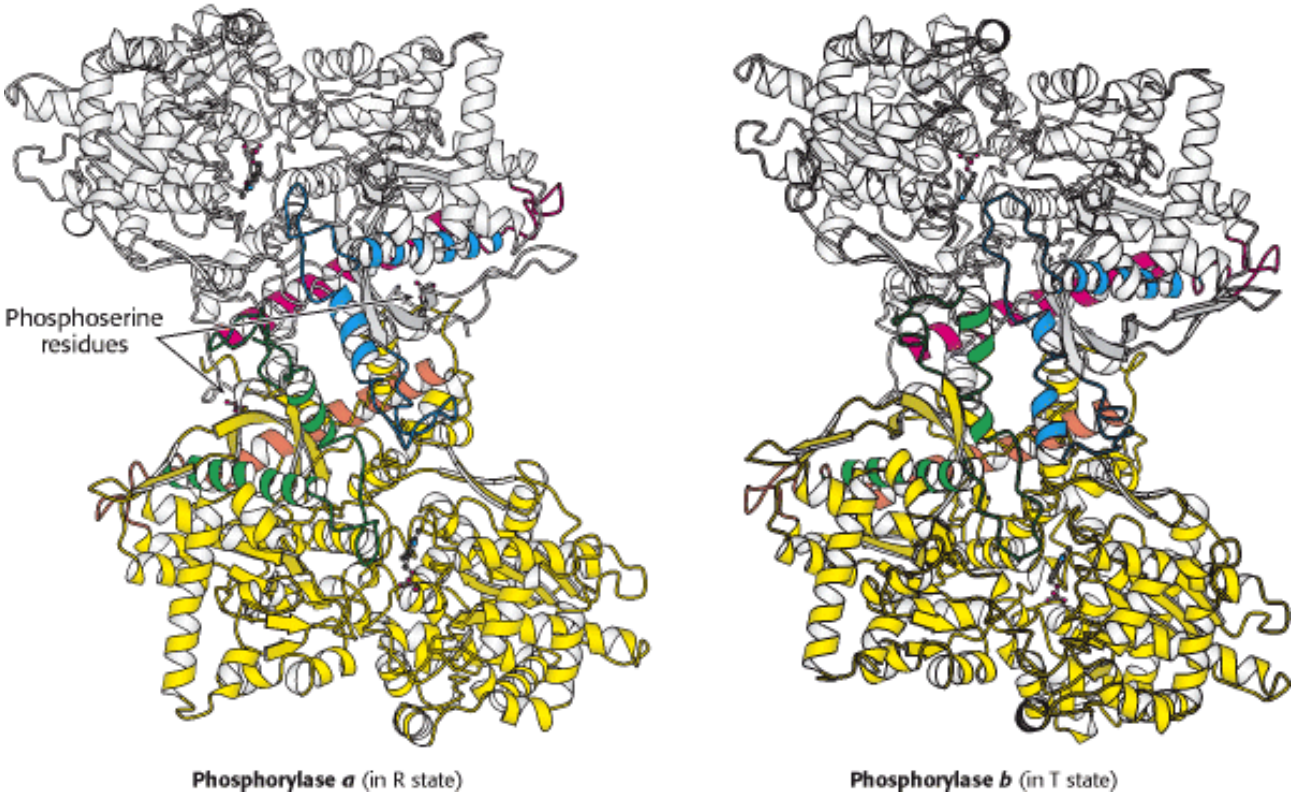


Figure 21.9. Structures of Phosphorylase A and Phosphorylase B . Phosphorylase *a* is phosphorylated on serine 14 of each subunit. This modification favors the structure of the more active R state. One subunit is shown in white, with helices and loops important for regulation shown in blue and red. The other subunit is shown in yellow, with the regulatory structures shown in orange and green. Phosphorylase *b* is not phosphorylated and exists predominantly in the T state.

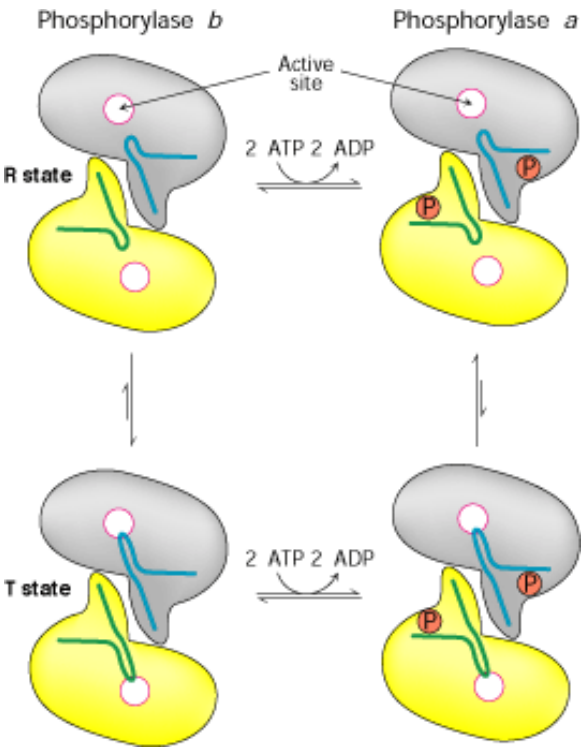


Figure 21.10. Phosphorylase Regulation. Both phosphorylase *b* and phosphorylase *a* exist as equilibria between an

active R state and a less-active T state. Phosphorylase *b* is usually inactive because the equilibrium favors the T state. Phosphorylase *a* is usually active because the equilibrium favors the R state. Regulatory structures are shown in blue and green.

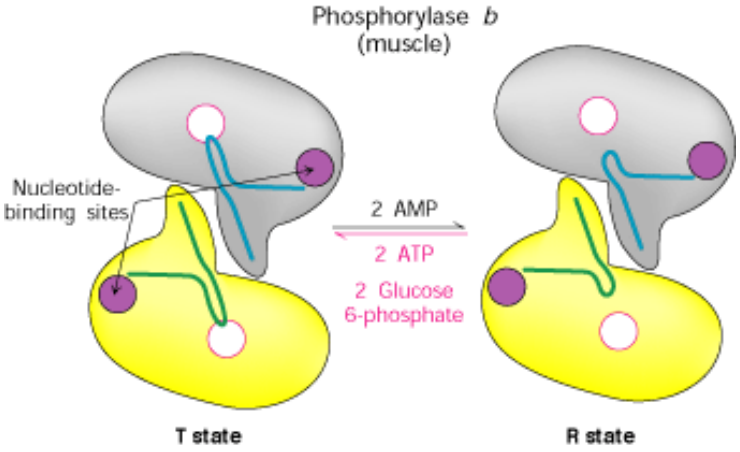


Figure 21.11. Allosteric Regulation of Muscle Phosphorylase. A low energy charge, represented by high concentrations of AMP, favors the transition to the R state.

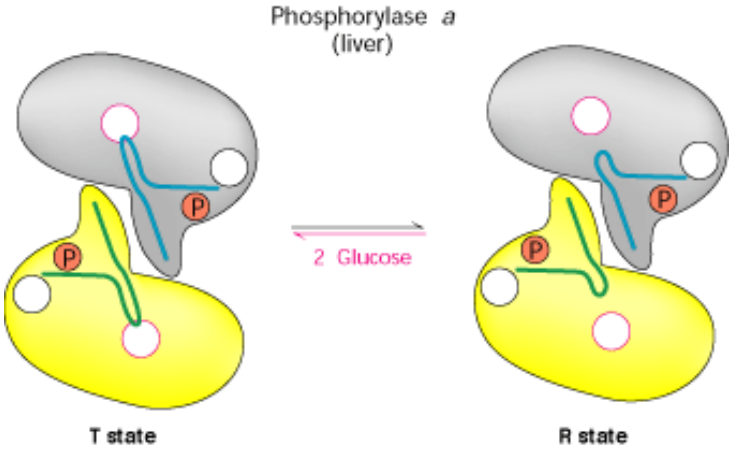


Figure 21.12. Allosteric Regulation of Liver Phosphorylase. The binding of glucose to phosphorylase *a* shifts the equilibrium to the T state and inactivates the enzyme. Thus, glycogen is not mobilized when glucose is already abundant.

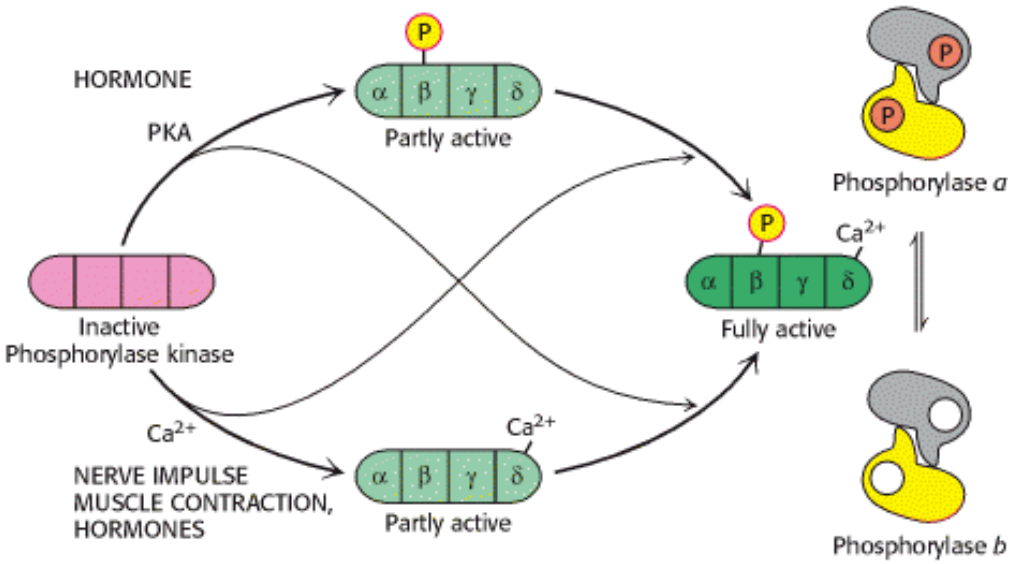


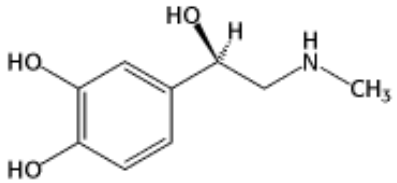
Figure 21.13. Activation of Phosphorylase Kinase. Phosphorylase kinase is activated by hormones that lead to the phosphorylation of the β subunit and by Ca^{2+} binding of the δ subunit. Both types of stimulation are required for maximal enzyme activity.

21.3. Epinephrine and Glucagon Signal the Need for Glycogen Breakdown

Protein kinase A activates phosphorylase kinase, which in turn activates glycogen phosphorylase. What activates protein kinase A? What is the signal that ultimately triggers an increase in glycogen breakdown?

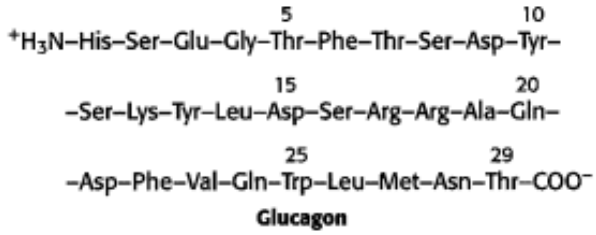
21.3.1. G Proteins Transmit the Signal for the Initiation of Glycogen Breakdown

Several hormones greatly affect glycogen metabolism. Glucagon and epinephrine trigger the breakdown of glycogen. Muscular activity or its anticipation leads to the release of *epinephrine (adrenaline)*, a catecholamine derived from tyrosine, from the adrenal medulla. Epinephrine markedly stimulates glycogen breakdown in muscle and, to a lesser extent, in the liver. The liver is more responsive to *glucagon*, a polypeptide hormone that is secreted by the α cells of the pancreas when the blood-sugar level is low. Physiologically, glucagon signifies the starved state.



Epinephrine

How do hormones trigger the breakdown of glycogen? We will briefly review this signal-transduction cascade, already discussed in Section 15.1 (Figure 21.14).



Glucagon

1. The signal molecules epinephrine and glucagon bind to specific 7TM receptors in the plasma membranes of muscle and liver cells, respectively. Epinephrine binds to the β-adrenergic receptor in muscle, whereas glucagon binds to the glucagon receptor. These binding events activate the α subunit of the heteromeric G_s protein. *A specific external signal has been transmitted into the cell through structural changes,* first in the receptor and then in the G protein.

2. The GTP-bound form of the α subunit of G_s activates adenylate cyclase, a transmembrane protein that catalyzes the formation of the secondary messenger cyclic AMP from ATP.

3. The elevated cytosolic level of cyclic AMP activates *protein kinase A* through the binding of cyclic AMP to the regulatory subunits, which then dissociate from the catalytic subunits. The free catalytic subunits are now active.

4. Protein kinase A phosphorylates the β subunit of phosphorylase kinase, which subsequently activates glycogen phosphorylase.

The cyclic AMP cascade highly amplifies the effects of hormones. Hence, the binding of a small number of hormone molecules to cell-surface receptors leads to the release of a very large number of sugar units. Indeed, the amplification is so large that much of the stored glycogen would be mobilized within seconds were it not for a counterregulatory system (Section 21.3.2).

The signal-transduction processes in the liver are more complex than those in muscle. Epinephrine can also elicit glycogen degradation in the liver. However, in addition to binding to the β-adrenergic receptor, it binds to the 7TM α-adrenergic receptor, which then activates phospholipase C and, hence, initiates the *phosphoinositide cascade* (Section 15.2). The consequent rise in the level of inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from endoplasmic reticulum stores. Recall that the δ subunit of phosphorylase kinase is the Ca^{2+} sensor calmodulin. Binding of Ca^{2+} to calmodulin leads to a partial activation of phosphorylase kinase. Stimulation by both glucagon and epinephrine leads to maximal mobilization of liver glycogen.

21.3.2. Glycogen Breakdown Must Be Capable of Being Rapidly Turned Off

There must be a way to shut down the high-gain system of glycogen breakdown quickly to prevent the wasteful depletion of glycogen after energy needs have been met. Indeed, another cascade leads to the dephosphorylation and inactivation of phosphorylase kinase and glycogen phosphorylase. Simultaneously, glycogen synthesis is activated.

The signal-transduction pathway leading to the activation of glycogen phosphorylase is shut down by the process already described for such pathways employing G proteins and cyclic AMP. The inherent GTPase activity of the G protein converts the bound GTP into GDP, thereby halting signal transduction. Cells also contain phosphodiesterase activity that converts cyclic AMP into AMP. Protein kinase A sets the stage for the shutdown of glycogen degradation by adding a phosphoryl group to the α subunit of phosphorylase kinase after first phosphorylating the β subunit. This addition of a phosphoryl group renders the enzyme a better substrate for dephosphorylation and consequent inactivation by the enzyme *protein phosphatase 1 (PP1).* Protein phosphatase 1 also removes the phosphoryl group from glycogen phosphorylase, converting the enzyme into the usually inactive *b* form.

21.3.3. The Regulation of Glycogen Phosphorylase Became More Sophisticated as the Enzyme Evolved

Analyses of the primary structures of glycogen phosphorylase from human beings, rats, *Dictyostelium* (slime mold), yeast, potatoes, and *E. coli* have enabled inferences to be made about the evolution of this important enzyme. The 16 residues that come into contact with glucose at the active site are identical in nearly all the enzymes. There is more variation but still substantial conservation of the 15 residues at the pyridoxal phosphate-binding site. Likewise, the glycogen-binding site is relatively well conserved in all the enzymes. The high degree of similarity of the active site, the pyridoxal phosphate-binding site and the glycogen-binding site shows that the catalytic mechanism has

been maintained throughout evolution.

Differences arise, however, when we compare the regulatory sites. The simplest type of regulation would be feedback inhibition by glucose 6-phosphate. Indeed, the glucose 6-phosphate regulatory site is highly conserved among the majority of the phosphorylases. The crucial amino acid residues that participate in regulation by phosphorylation and nucleotide binding are well conserved only in the mammalian enzymes. Thus, this level of regulation was a later evolutionary acquisition.

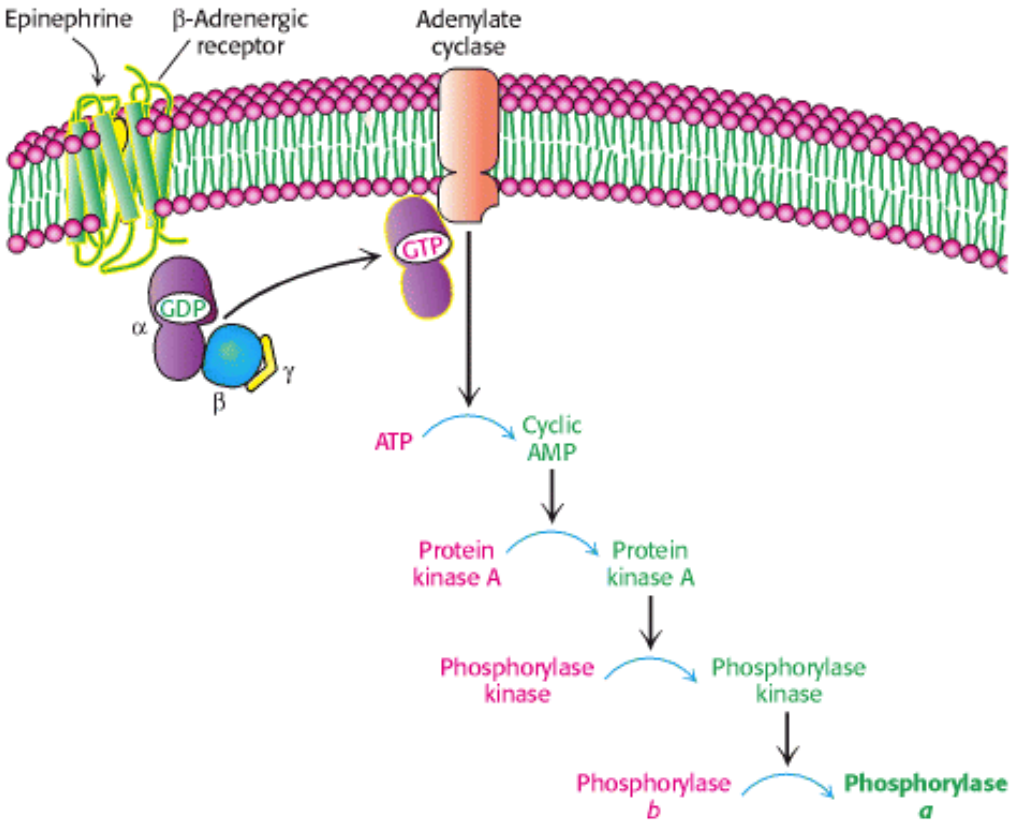


Figure 21.14. Regulatory Cascade for Glycogen Breakdown. Glycogen degradation is stimulated by hormone binding to 7TM receptors. Hormone binding initiates a G-protein-dependent signal-transduction pathway that results in the phosphorylation and activation of glycogen phosphorylase.

21.4. Glycogen Is Synthesized and Degraded by Different Pathways

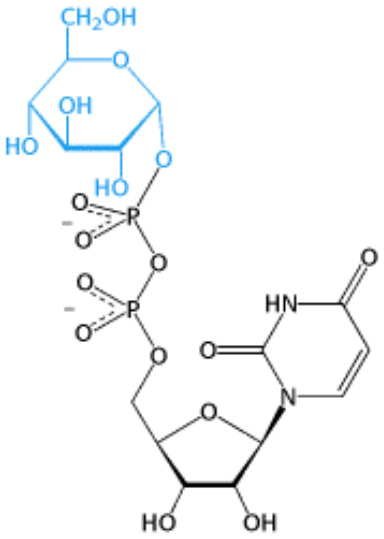
Conceptual Insights, Overview of Carbohydrate and Fatty Acid Metabolism. View this media module to better understand how glycogen metabolism fits in with other energy storage and utilization pathways (glycolysis, citric acid cycle, pentose phosphate pathway, and fatty acid metabolism).

As we have seen in glycolysis and gluconeogenesis, biosynthetic and degradative pathways rarely operate by precisely the same reactions in the forward and reverse directions. Glycogen metabolism provided the first known example of this important principle. *Separate pathways afford much greater flexibility, both in energetics and in control.*

In 1957, Luis Leloir and his coworkers showed that glycogen is synthesized by a pathway that utilizes *uridine diphosphate glucose (UDP-glucose)* rather than glucose 1-phosphate as the activated glucose donor.

$$\text{Synthesis: Glycogen}_n + \text{UDP-glucose} \longrightarrow \text{glycogen}_{n+1} + \text{UDP}$$

$$\text{Degradation: Glycogen}_{n+1} + \text{P}_i \longrightarrow \text{glycogen}_n + \text{glucose 1-phosphate}$$

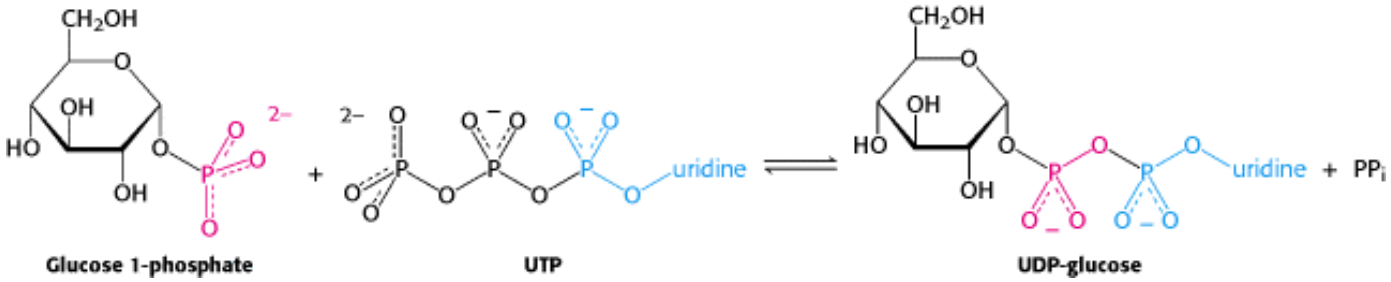


Uridine diphosphate glucose (UDP-glucose)

21.4.1. UDP-Glucose Is an Activated Form of Glucose

UDP-glucose, the glucose donor in the biosynthesis of glycogen, is an *activated form of glucose*, just as ATP and acetyl CoA are activated forms of orthophosphate and acetate, respectively. The C-1 carbon atom of the glucosyl unit of UDP-glucose is activated because its hydroxyl group is esterified to the diphosphate moiety of UDP.

UDP-glucose is synthesized from glucose 1-phosphate and uridine triphosphate (UTP) in a reaction catalyzed by *UDP-glucose pyrophosphorylase*. The pyrophosphate liberated in this reaction comes from the outer two phosphoryl residues of UTP.



Glucose 1-phosphate **UTP** **UDP-glucose**

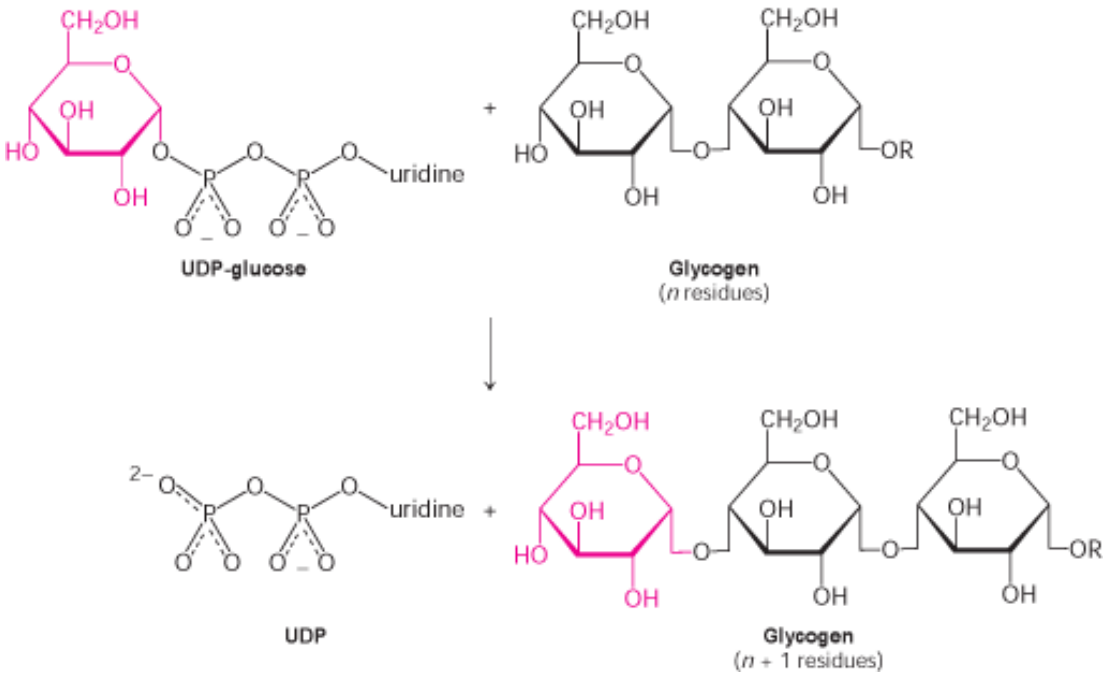
This reaction is readily reversible. However, pyrophosphate is rapidly hydrolyzed in vivo to orthophosphate by an inorganic pyrophosphatase. The essentially irreversible hydrolysis of pyrophosphate drives the synthesis of UDP-glucose.

$$\begin{aligned} \text{Glucose 1-phosphate} + \text{UTP} &\rightleftharpoons \text{UDP-glucose} + \text{PP}_i \\ \text{PP}_i + \text{H}_2\text{O} &\longrightarrow 2\,\text{P}_i \\ \hline \text{Glucose 1-phosphate} + \text{UTP} + \text{H}_2\text{O} &\longrightarrow \text{UDP-glucose} + 2\,\text{P}_i \end{aligned}$$

The synthesis of UDP-glucose exemplifies another recurring theme in biochemistry: *many biosynthetic reactions are driven by the hydrolysis of pyrophosphate.*

21.4.2. Glycogen Synthase Catalyzes the Transfer of Glucose from UDP-Glucose to a Growing Chain

New glucosyl units are added to the nonreducing terminal residues of glycogen. The activated glucosyl unit of UDP-glucose is transferred to the hydroxyl group at a C-4 terminus of glycogen to form an α-1,4-glycosidic linkage. In elongation, UDP is displaced by the terminal hydroxyl group of the growing glycogen molecule. This reaction is catalyzed by *glycogen synthase, the key regulatory enzyme in glycogen synthesis.*



Glycogen synthase can add glucosyl residues only if the polysaccharide chain already contains more than four residues. Thus, glycogen synthesis requires a *primer*. This priming function is carried out by *glycogenin*, a protein composed of two identical 37-kd subunits, each bearing an oligosaccharide of α-1,4-glucose units. Carbon 1 of the first unit of this chain, the reducing end, is covalently attached to the phenolic hydroxyl group of a specific tyrosine in each glycogenin subunit. How is this chain formed? Each subunit of glycogenin catalyzes the addition of eight glucose units to its partner in the glycogenin dimer. UDP-glucose is the donor in this autoglycosylation. At this point, glycogen synthase takes over to extend the glycogen molecule.

21.4.3. A Branching Enzyme Forms α-1,6 Linkages

Glycogen synthase catalyzes only the synthesis of α-1,4 linkages. Another enzyme is required to form the α-1,6 linkages that make glycogen a branched polymer. Branching occurs after a number of glucosyl residues are joined in α-1,4 linkage by glycogen synthase. A branch is created by the breaking of an α-1,4 link and the formation of an α-1,6 link: this reaction is different from debranching. A block of residues, typically 7 in number, is transferred to a more interior site. The *branching enzyme* that catalyzes this reaction is quite exacting. The block of 7 or so residues must include the nonreducing terminus and come from a chain at least 11 residues long. In addition, the new branch point must be at least 4 residues away from a preexisting one.

Branching is important because it increases the solubility of glycogen. Furthermore, branching creates a large number of terminal residues, the sites of action of glycogen phosphorylase and synthase (Figure 21.15). Thus, *branching increases the rate of glycogen synthesis and degradation.*

Glycogen branching requires a single transferase activity. Glycogen debranching requires two enzyme activities: a transferase and an α-1,6 glucosidase. Sequence analysis suggests that the two transferases and, perhaps, the α-1,6

glucosidase are members of the same enzyme family, termed the α *-amylase family*. Such an enzyme catalyzes a reaction by forming a covalent intermediate attached to a conserved aspartate residue (Figure 21.16). Thus, the branching enzyme appears to function through the transfer of a chain of glucose molecules from an α-1,4 linkage to an aspartate residue on the enzyme and then from this site to a more interior location on the glycogen molecule to form an α-1,6 linkage.

21.4.4. Glycogen Synthase Is the Key Regulatory Enzyme in Glycogen Synthesis

The activity of glycogen synthase, like that of phosphorylase, is regulated by covalent modification. Glycogen synthase is phosphorylated at multiple sites by protein kinase A and several other kinases. The resulting alteration of the charges in the protein lead to its inactivation (Figure 21.17). *Phosphorylation has opposite effects on the enzymatic activities of glycogen synthase and phosphorylase.* Phosphorylation converts the active *a* form of the synthase into a usually inactive *b* form. The phosphorylated *b* form requires a high level of the allosteric activator glucose 6-phosphate for activity, whereas the *a* form is active whether or not glucose 6-phosphate is present.

21.4.5. Glycogen Is an Efficient Storage Form of Glucose

What is the cost of converting glucose 6-phosphate into glycogen and back into glucose 6-phosphate? The pertinent reactions have already been described, except for reaction 5, which is the regeneration of UTP. ATP phosphorylates UDP in a reaction catalyzed by *nucleoside diphosphokinase.*

$$\text{Glucose 6-phosphate} \longrightarrow \text{glucose 1-phosphate} \quad (1)$$

$$\text{Glucose 1-phosphate} + \text{UTP} \longrightarrow \text{UDP-glucose} + \text{PP}_i \quad (2)$$

$$\text{PP}_i + \text{H}_2\text{O} \longrightarrow 2\,\text{P}_i \quad (3)$$

$$\text{UDP-glucose} + \text{glycogen}_n \longrightarrow \text{glycogen}_{n+1} + \text{UDP} \quad (4)$$

$$\text{UDP} + \text{ATP} \longrightarrow \text{UTP} + \text{ADP} \quad (5)$$

$$\text{Sum: Glucose 6-phosphate} + \text{ATP} + \text{glycogen}_n + \text{H}_2\text{O} \longrightarrow \text{glycogen}_{n+1} + \text{ADP} + 2\,\text{P}_i$$

Thus, one ATP is hydrolyed incorporating glucose 6-phosphate into glycogen. The energy yield from the breakdown of glycogen is highly efficient. About 90% of the residues are phosphorolytically cleaved to glucose 1-phosphate, which is converted at no cost into glucose 6-phosphate. The other 10% are branch residues, which are hydrolytically cleaved. One molecule of ATP is then used to phosphorylate each of these glucose molecules to glucose 6-phosphate. The complete oxidation of glucose 6-phosphate yields about 31 molecules of ATP, and storage consumes slightly more than one molecule of ATP per molecule of glucose 6-phosphate; so *the overall efficiency of storage is nearly 97%.*

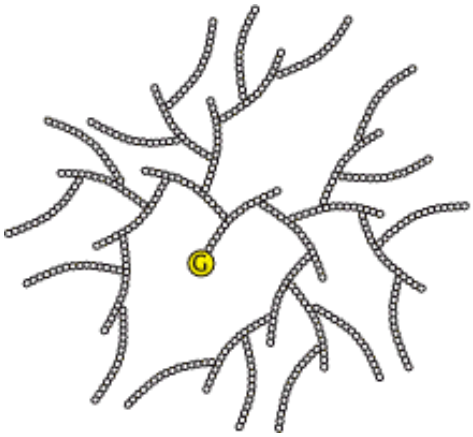


Figure 21.15. Cross Section of a Glycogen Molecule. The component labeled G is glycogenin.

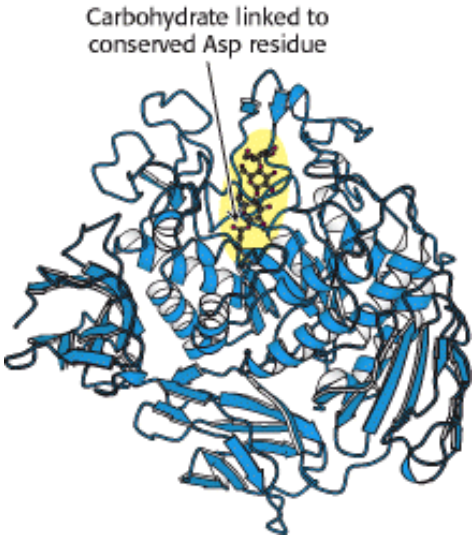


Figure 21.16. Structure of Glycogen Transferase. A conserved aspartate residue forms a covalent intermediate with a chain of glucose molecules.

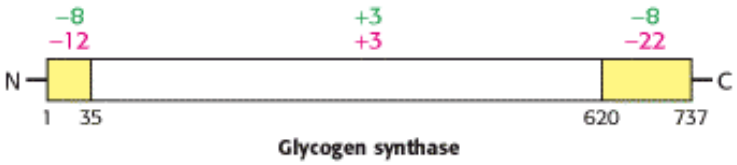


Figure 21.17. Charge Distribution of Glycogen Synthase. Glycogen synthase has a highly asymmetric charge distribution. Phosphorylation markedly changes the net charge of the amino- and carboxyl-terminal regions (yellow) of the enzyme. The net charge of these regions and the interior of the enzyme before and after complete phosphorylation are shown in green and red, respectively. [After M. F. Browner, K. Nakano, A. G. Bang, and R. J. Fletterick. *Proc. Natl. Acad. Sci. USA* 86(1989):1443.]

21.5. Glycogen Breakdown and Synthesis Are Reciprocally Regulated

We now return to the regulation of glycogen metabolism with a knowledge of both degradation and synthesis. *Glycogen breakdown and synthesis are reciprocally regulated by a hormone-triggered cAMP cascade acting through protein kinase A* (Figure 21.18). In addition to phosphorylating and activating phosphorylase kinase, protein kinase A adds a phosphoryl group to glycogen synthase, which leads to a *decrease* in enzymatic activity. This important control mechanism prevents glycogen from being synthesized at the same time that it is being broken down. How is the enzymatic activity reversed so that glycogen breakdown halts and glycogen synthesis begins?

21.5.1. Protein Phosphatase 1 Reverses the Regulatory Effects of Kinases on Glycogen Metabolism

The changes in enzymatic activity produced by protein kinases are reversed by *protein phosphatases*. The hydrolysis of phosphorylated serine and threonine residues in proteins is catalyzed by protein phosphatases. One enzyme, termed *protein phosphatase 1, plays key roles in regulating glycogen metabolism.* PP1 inactivates phosphorylase kinase and phosphorylase *a* by dephosphorylating these enzymes. PP1 decreases the rate of glycogen breakdown: it reverses the effects of the phosphorylation cascade. Moreover, PP1 also removes the phosphoryl group from glycogen synthase *b* to convert it into the much more active *a* form. Hence, PP1 accelerates glycogen synthesis. PP1 is yet another molecular device for coordinating carbohydrate storage.

The complete complex of PP1 consists of three components: PP1 itself, a 37-kd catalytic subunit; a 123-kd R_{Gl} subunit that confers high affinity for glycogen; and inhibitor 1, a small regulatory subunit that, when phosphorylated, inhibits PP1. The importance of the R_{Gl} subunit is that it brings PP1, which is active only when associated with glycogen molecules, into proximity with its substrates.

How is the phosphatase activity of PP1 itself regulated? Consider the case in which glycogen degradation is predominant (Figure 21.19). In this case, PKA is active. Two components of PP1 are themselves substrates for protein kinase A. Phosphorylation of the R_{Gl} component by protein kinase A prevents R_{Gl} from binding the catalytic subunit of PP1. Consequently, activation of the cAMP cascade leads to the inactivation of PP1 because it can no longer bind its substrates. Phosphorylation of inhibitor 1 by protein kinase A blocks catalysis by PP1. Thus, when glycogen degradation is switched on by cAMP, the accompanying phosphorylation of inhibitor 1 keeps phosphorylase in the active *a* form and glycogen synthase in the inactive *b* form. The epinephrine-induced phosphorylation of the R_{Gl} subunit and inhibitor 1 are complementary devices for sustaining glycogen degradation.

21.5.2. Insulin Stimulates Glycogen Synthesis by Activating Protein Phosphatase 1

How is glycogen synthesis stimulated? As stated earlier, the presence of glucagon signifies the starved state and initiates glycogen breakdown while inhibiting glycogen synthesis. When blood-glucose levels are high, *insulin stimulates the synthesis of glycogen by triggering a pathway that activates protein phosphatase 1* (Figure 21.20). The first step in the action of insulin is its binding to a receptor tyrosine kinase in the plasma membrane. Multiple *phosphorylations* again serve as the instigation for a regulatory wave of *dephosphorylations*. The binding of insulin to its receptor leads to the activation of an *insulin-sensitive protein kinase* that phosphorylates the R_{Gl} subunit of PP1 at a site *different* from that modified by protein kinase A. This phosphorylation leads to the association of the R_{Gl} subunit with PP1 and the glycogen molecule. The consequent dephosphorylation of glycogen synthase, phosphorylase kinase, and phosphorylase promotes glycogen synthesis and blocks its degradation. Once again we see that *glycogen synthesis and breakdown are coordinately controlled.*

21.5.3. Glycogen Metabolism in the Liver Regulates the Blood-Glucose Level

After a meal rich in carbohydrates, blood-glucose levels rise, leading to an increase in glycogen synthesis in the liver. Although insulin is the primary signal for glycogen synthesis, other, nonhormonal mechanisms also function in the liver. One signal is the concentration of glucose in the blood, which normally ranges from about 80 to 120 mg per 100 ml (4.4 – 6.7 mM). The liver senses the concentration of glucose in the blood and takes up or releases glucose accordingly. The amount of liver phosphorylase *a* decreases rapidly when glucose is infused (Figure 21.21). After a lag period, the amount of glycogen synthase *a* increases, which results in the synthesis of glycogen. In fact, *phosphorylase* a *is the glucose sensor in liver cells.* The binding of glucose to phosphorylase *a* shifts its allosteric equilibrium from the active R form to the inactive T form. This conformational change *renders the phosphoryl group on serine 14 a substrate for protein phosphatase 1.* It is significant that PP1 binds tightly to phosphorylase *a* but acts catalytically only when glucose induces the transition to the T form. Recall that the R ⟷ T transition of muscle phosphorylase *a* is unaffected by glucose and is thus unaffected by the rise in blood-glucose levels (Section 21.2.2).

How does glucose activate glycogen synthase? Phosphorylase *b*, in contrast with phosphorylase *a*, does not bind the phosphatase. Consequently, the conversion of *a* into *b* is accompanied by the *release of PP1, which is then free to activate glycogen synthase* (Figure 21.22). Removal of the phosphoryl group of inactive glycogen synthase *b* converts it into the active *a* form. Initially, there are about 10 phosphorylase *a* molecules per molecule of phosphatase. Hence, *the activity of glycogen synthase begins to increase only after most of phosphorylase* a *is converted into* b. This remarkable glucose-sensing system depends on three key elements: (1) communication between the serine phosphate and the allosteric site for glucose, (2) the use of PP1 to inactivate phosphorylase and activate glycogen synthase, and (3) the binding of the phosphatase to phosphorylase *a* to prevent the premature activation of glycogen synthase.

21.5.4. A Biochemical Understanding of Glycogen-Storage Diseases Is Possible


Edgar von Gierke described the first glycogen-storage disease in 1929. A patient with this disease has a huge abdomen caused by a *massive enlargement of the liver.* There is a pronounced *hypoglycemia* between meals. Furthermore, the blood-glucose level does not rise on administration of epinephrine and glucagon. An infant with this glycogen-storage disease may have convulsions because of the low blood-glucose level.

The enzymatic defect in von Gierke disease was elucidated in 1952 by Carl and Gerty Cori. They found that *glucose 6-phosphatase is missing from the liver of a patient with this disease.* This was the first demonstration of an inherited deficiency of a liver enzyme. The liver glycogen is normal in structure but present in abnormally large amounts. The absence of glucose 6-phosphatase in the liver causes hypoglycemia because glucose cannot be formed from glucose 6-phosphate. This phosphorylated sugar does not leave the liver, because it cannot cross the plasma membrane. The presence of excess glucose 6-phosphate triggers an increase in glycolysis in the liver, leading to a high level of lactate and pyruvate in the blood. Patients who have von Gierke disease also have an increased dependence on fat metabolism. This disease can also be produced by a mutation in the gene that encodes the *glucose 6-phosphate transporter.* Recall that glucose 6-phosphate must be transported into the lumen of the endoplasmic reticulum to be hydrolyzed by phosphatase (Section 16.3.5). Mutations in the other three essential proteins of this system can likewise lead to von Gierke disease.

Seven other glycogen-storage diseases have been characterized (Table 21.1). In Pompe disease (type II), lysosomes become engorged with glycogen because they lack α-1,4-glucosidase, a hydrolytic enzyme confined to these organelles (Figure 21.23). The Coris elucidated the biochemical defect in another glycogen-storage disease (type III), which cannot be distinguished from von Gierke disease (type I) by physical examination alone. In type III disease, the structure of liver and muscle glycogen is abnormal and the amount is markedly increased. Most striking, the outer branches of the glycogen are very short. *Patients having this type lack the debranching enzyme (α-1,6-glucosidase),* and so only the outermost branches of glycogen can be effectively utilized. Thus, only a small fraction of this abnormal glycogen is functionally active as an accessible store of glucose.

A defect in glycogen metabolism confined to muscle is found in McArdle disease (type V). *Muscle phosphorylase activity is absent,* and the patient's capacity to perform strenuous exercise is limited because of painful muscle cramps. The patient is otherwise normal and well developed. Thus, effective utilization of muscle glycogen is not essential for life. The results of phosphorus-31 nuclear magnetic resonance studies of these patients have been very informative. The

pH of skeletal muscle cells of normal people drops during strenuous exercise because of the production of lactate. In contrast, the muscle cells of patients with McArdle disease become more alkaline during exercise because of the breakdown of creatine phosphate (Section 14.1.5). Lactate does not accumulate in these patients because the glycolytic rate of their muscle is much lower than normal; their glycogen cannot be mobilized. The results of NMR studies have also shown that the painful cramps in this disease are correlated with high levels of ADP (Figure 21.24). NMR spectroscopy is a valuable, noninvasive technique for assessing dietary and exercise therapy for this disease.

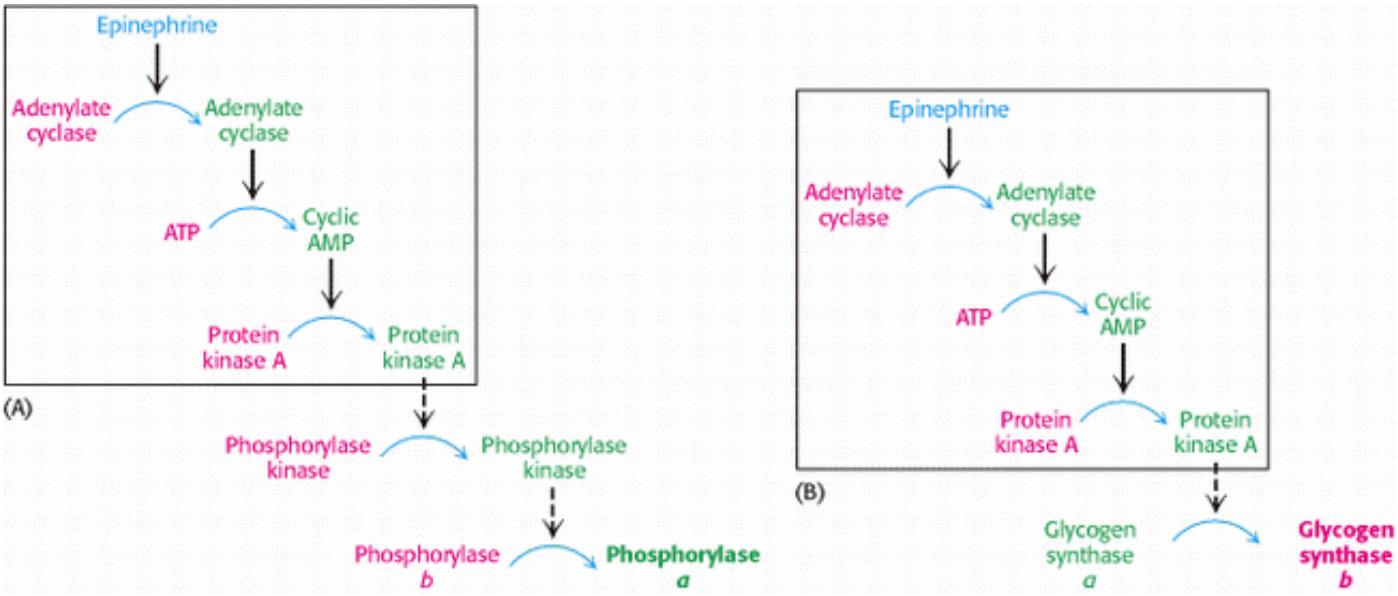


Figure 21.18. Coordinate Control of Glycogen Metabolism. Glycogen metabolism is regulated, in part, by hormone-triggered cyclic AMP cascades: (A) glycogen degradation; (B) glycogen synthesis. Inactive forms are shown in red, and active ones in green. The sequence of reactions leading to the activation of protein kinase A is the same in the regulation of glycogen degradation and synthesis. Phosphorylase kinase also inactivates glycogen synthase.

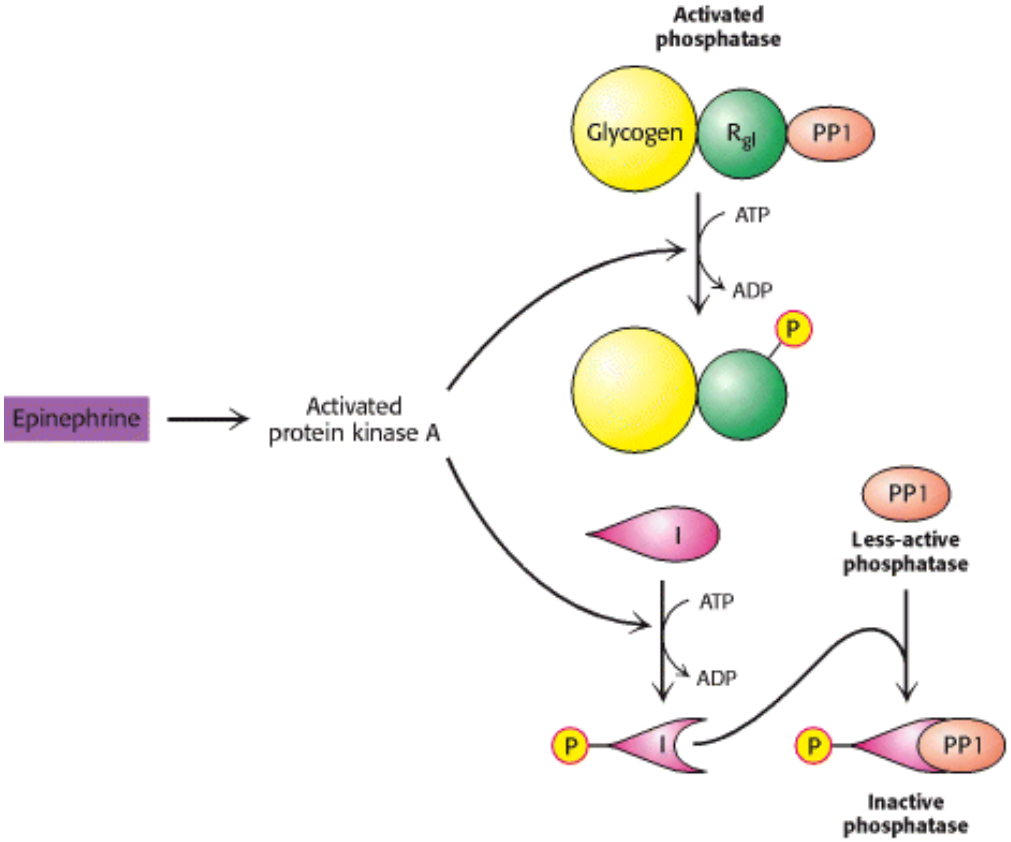


Figure 21.19. Regulation of Protein Phosphatase 1 (PP1). Phosphorylation of R_{Gl} by protein kinase A dissociates the catalytic subunit from the glycogen particle and hence the PP1 substrates. Inhibition is complete when the inhibitor subunit (I) is phosphorylated and binds to PP1 to inactivate it.

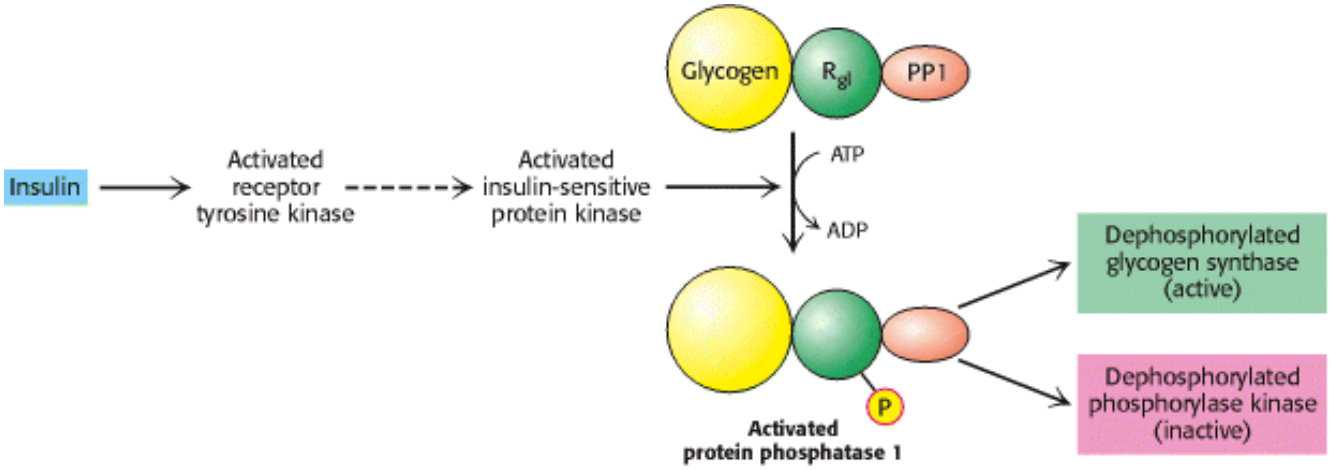


Figure 21.20. Insulin Activates Protein Phosphatase 1. Insulin triggers a cascade leading to the activation of protein phosphatase 1, which results in the stimulation of glycogen synthesis and inhibition of its breakdown. The activated receptor tyrosine kinase switches on a putative master kinase that phosphorylates the insulin-sensitive protein kinase. In turn, the glycogen-targeting subunit (R_{Gl} subunit) of the phosphatase is phosphorylated, which activates the enzyme. [After P. Dent, A. Lavoinne, S. Nakielny, F. B. Caudwell, P. Watt, and P. Cohen. *Nature* 348(1990):306.]

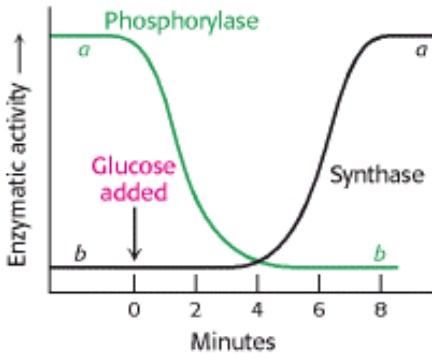


Figure 21.21. Blood Glucose Regulates Liver Glycogen Metabolism. The infusion of glucose into the bloodstream leads to the inactivation of phosphorylase, followed by the activation of glycogen synthase, in the liver. [After W. Stalmans, H. De Wulf, L. Hue, and H.-G. Hers. *Eur. J. Biochem.* 41(1974):127.]

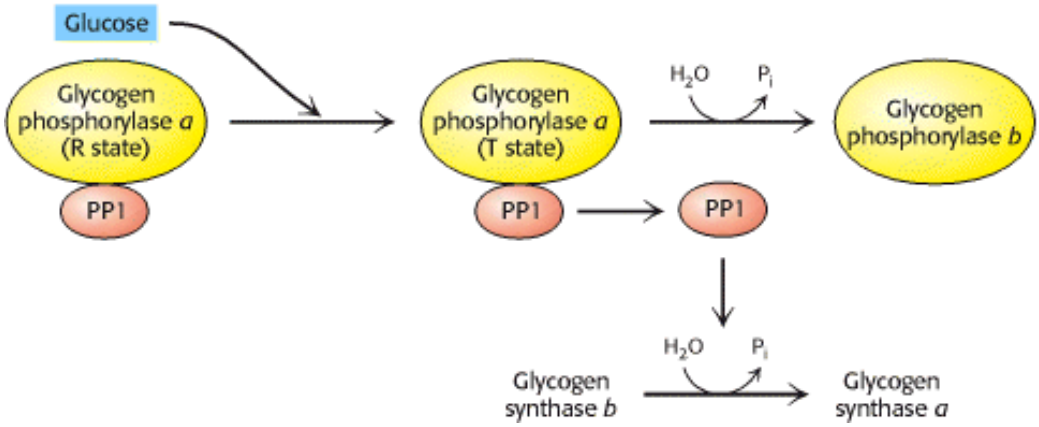


Figure 21.22. Glucose Regulation of Liver Glycogen Metabolism. Glucose binds to and inhibits glycogen phosphorylase *a* in the liver, leading to the dissociation and activation of protein phosphatase 1 (PP1) from glycogen phosphorylase *a*. The free PP1 dephosphorylates glycogen phosphorylase *a* and glycogen synthase *b*, leading to the inactivation of glycogen breakdown and the activation of glycogen synthesis.

Table 21.1. Glycogen-storage diseases

Type	Defective enzyme	Organ affected	Glycogen in the affected organ	Clinical features
I Von Gierke disease	Glucose 6-phosphatase or transport system	Liver and kidney	Increased amount; normal structure.	Massive enlargement of the liver. Failure to thrive. Severe hypoglycemia, ketosis, hyperuricemia, hyperlipemia.
II Pompe disease	α-1,4-Glucosidase (lysosomal)	All organs	Massive increase in amount; normal structure.	Cardiorespiratory failure causes death, usually before age 2.
III Cori disease	Amylo-1,6-glucosidase (debranching enzyme)	Muscle and liver	Increased amount; short outer branches.	Like type I, but milder course.

IV Andersen disease	Branching enzyme (α-1,4 $\longrightarrow$ α-1,6)	Liver and spleen	Normal amount; very long outer branches.	Progressive cirrhosis of the liver. Liver failure causes death, usually before age 2.
V McArdle disease	Phosphorylase	Muscle	Moderately increased amount; normal structure.	Limited ability to perform strenuous exercise because of painful muscle cramps. Otherwise patient is normal and well developed.
VI Hers disease	Phosphorylase	Liver	Increased amount.	Like type I, but milder course.
VII	Phosphofructokinase	Muscle	Increased amount; normal structure.	Like type V.
VIII	Phosphorylase kinase	Liver	Increased amount; normal structure.	Mild liver enlargement. Mild hypoglycemia.

Note: Types I through VII are inherited as autosomal recessives. Type VIII is sex linked.

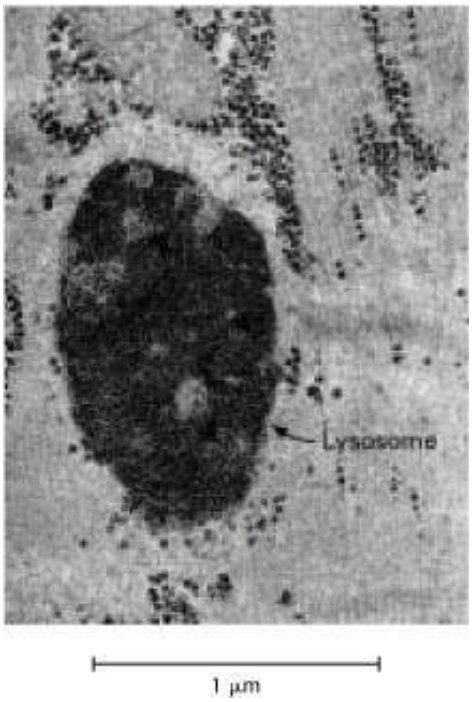


Figure 21.23. Glycogen-Engorged Lysosome. This electron micrograph shows skeletal muscle from an infant with type II glycogen-storage disease (Pompe disease). The lysosomes are filled with glycogen because of a deficiency in α-1,4-glucosidase, a hydrolytic enzyme confined to lysosomes. The amount of glycogen in the cytosol is normal. [From H.-G. Hers and F. Van Hoof, Eds. *Lysosomes and Storage Diseases* (Academic Press, 1973), p. 205.]

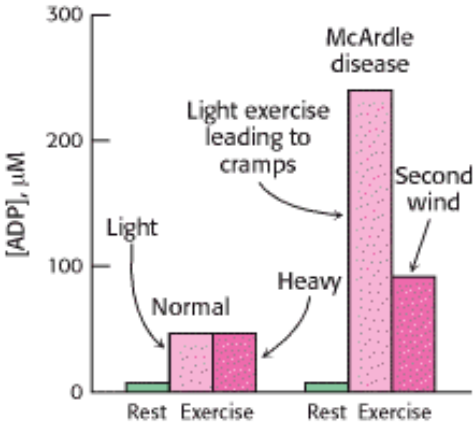


Figure 21.24. NMR Study of Human Arm Muscle. The level of ADP during exercise increases much more in a patient with McArdle glycogen-storage disease (type V) than in normal controls. [After G. K. Radda. *Biochem. Soc. Trans.* 14 (1986):522.]

Summary

Glycogen, a readily mobilized fuel store, is a branched polymer of glucose residues. Most of the glucose units in glycogen are linked by α-1,4 glycosidic bonds. At about every tenth residue, a branch is created by an α-1,6-glycosidic bond. Glycogen is present in large amounts in muscle cells and in liver cells, where it is stored in the cytoplasm in the form of hydrated granules.

Glycogen Breakdown Requires the Interplay of Several Enzymes

Most of the glycogen molecule is degraded to glucose 1-phosphate by the action of glycogen phosphorylase, the key enzyme in glycogen breakdown. The glycosidic linkage between C-1 of a terminal residue and C-4 of the adjacent one is split by orthophosphate to give glucose 1-phosphate, which can be reversibly converted into glucose 6-phosphate. Branch points are degraded by the concerted action of an oligosaccharide transferase and an α-1,6-glucosidase.

Phosphorylase Is Regulated by Allosteric Interactions and Reversible Phosphorylation

Phosphorylase is regulated by allosteric effectors and reversible covalent modifications. Phosphorylase *b*, which is usually inactive, is converted into active phosphorylase *a* by the phosphorylation of a single serine residue in each subunit. This reaction is catalyzed by phosphorylase kinase. The *b* form in muscle can also be activated by the binding of AMP, an effect antagonized by ATP and glucose 6-phosphate. The *a* form in the liver is inhibited by glucose. The AMP-binding sites and phosphorylation sites are located at the subunit interface. In muscle, phosphorylase is activated to generate glucose for use inside the cell as a fuel for contractile activity. In contrast, liver phosphorylase is activated to liberate glucose for export to other organs, such as skeletal muscle and the brain.

Epinephrine and Glucagon Signal the Need for Glycogen Breakdown

Epinephrine and glucagon stimulate glycogen breakdown through specific 7TM receptors. Muscle is the primary target of epinephrine, whereas the liver is responsive to glucagon. Both signal molecules initiate a kinase cascade that leads to the activation of glycogen phosphorylase.

Glycogen Is Synthesized and Degraded by Different Pathways

Glycogen is synthesized by a different pathway from that of glycogen breakdown. UDP-glucose, the activated intermediate in glycogen synthesis, is formed from glucose 1-phosphate and UTP. Glycogen synthase catalyzes the transfer of glucose from UDP-glucose to the C-4 hydroxyl group of a terminal residue in the growing glycogen molecule. Synthesis is primed by glycogenin, an autoglycosylating protein that contains a covalently attached oligosaccharide unit on a specific tyrosine residue. A branching enzyme converts some of the α-1,4 linkages into α-1,6 linkages to increase the number of ends so that glycogen can be made and degraded more rapidly.

Glycogen Breakdown and Synthesis Are Reciprocally Regulated

Glycogen synthesis and degradation are coordinated by several amplifying reaction cascades. Epinephrine and glucagon stimulate glycogen breakdown and inhibit its synthesis by increasing the cytosolic level of cyclic AMP, which activates protein kinase A. Elevated cytosolic Ca^{2+} levels stimulate glycogen degradation by activating phosphorylase kinase. Hence, muscle contraction and calcium-mobilizing hormones promote glycogen breakdown.

The glycogen-mobilizing actions of PKA are reversed by protein phosphatase 1, which is regulated by several hormones. Epinephrine inhibits this phosphatase by blocking its attachment to glycogen molecules and by turning on an inhibitor. Insulin, in contrast, activates this phosphatase by triggering a cascade that phosphorylates the glycogen-targeting subunit of this enzyme. Hence, glycogen synthesis is decreased by epinephrine and increased by insulin. Glycogen synthase and phosphorylase are also regulated by noncovalent allosteric interactions. In fact, phosphorylase is a key part of the glucose-sensing system of liver cells. Glycogen metabolism exemplifies the power and precision of reversible phosphorylation in regulating biological processes.

Key Terms

glycogen phosphorylase

phosphorolysis

pyridoxal phosphate

phosphorylase kinase

protein kinase A

calmodulin

epinephrine (adrenaline)

glucagon

uridine diphosphate glucose (UDPglucose)

glycogen synthase

glycogenin

protein phosphatase 1

insulin

Problems

1. *Carbohydrate conversion.* Write a balanced equation for the formation of glycogen from galactose.

See answer

2. *If a little is good, a lot is better.* α-Amylose is an unbranched glucose polymer. Why would this polymer not be as effective a storage form for glucose as glycogen?

See answer

3. *Telltale products.* A sample of glycogen from a patient with liver disease is incubated with orthophosphate, phosphorylase, the transferase, and the debranching enzyme (α-1,6-glucosidase). The ratio of glucose 1-phosphate to glucose formed in this mixture is 100. What is the most likely enzymatic deficiency in this patient?

See answer

4. *Excessive storage.* Suggest an explanation for the fact that the amount of glycogen in type I glycogen-storage disease (von Gierke disease) is increased.

See answer

5. *A shattering experience.* Crystals of phosphorylase *a* grown in the presence of glucose shatter when a substrate such as glucose 1-phosphate is added. Why?

See answer

6. *Recouping an essential phosphoryl.* The phosphoryl group on phosphoglucomutase is slowly lost by hydrolysis. Propose a mechanism that utilizes a known catalytic intermediate for restoring this essential phosphoryl group. How might this phosphoryl donor be formed?

See answer

7. *Hydrophobia.* Why is water excluded from the active site of phosphorylase? Predict the effect of a mutation that allows water molecules to enter.

See answer

8. *Removing all traces.* In human liver extracts, the catalytic activity of glycogenin was detectable only after treatment with α-amylase (Section 11.2.2). Why was α-amylase necessary to reveal the glycogenin activity?

See answer

9. *Two in one.* A single polypeptide chain houses the transferase and debranching enzyme. Cite a potential advantage of this arrangement.

See answer

10. *How did they do that?* A strain of mice has been developed that lack the enzyme phosphorylase kinase. Yet, after strenuous exercise, the glycogen stores of a mouse of this strain are depleted. Explain how this is possible.

See answer

11. *Metabolic mutants.* Predict the major consequence of each of the following mutations:

(a) Loss of the AMP-binding site in muscle phosphorylase.

(b) Mutation of Ser 14 to Ala 14 in liver phosphorylase.

(c) Overexpression of phosphorylase kinase in the liver.

(d) Loss of the gene that encodes inhibitor 1 of protein phosphatase 1.

(e) Loss of the gene that encodes the glycogen-targeting subunit of protein phosphatase 1.

(f) Loss of the gene that encodes glycogenin.

See answer

12. *More metabolic mutants.* Briefly, predict the major consequences of each of the following mutations affecting glycogen utilization.

(a) Loss of GTPase activity of the G protein α subunit.

(b) Loss of the gene that encodes inhibitor 1 of protein phosphatase 1.

(c) Loss of phosphodiesterase activity.

See answer

13. *Multiple phosphorylation.* Protein kinase A activates muscle phosphorylase kinase by rapidly phosphorylating its β subunits. The α subunits of phosphorylase kinase are then slowly phosphorylated, which makes the α and β subunits susceptible to the action of protein phosphatase 1. What is the functional significance of the slow phosphorylation of α?

See answer

14. *The wrong switch.* What would be the consequences to glycogen mobilization of a mutation in phosphorylase kinase that leads to the phosphorylation of the α-subunit prior to that of the β subunit?

See answer

Mechanism Problem

15. *Family resemblance.* Propose mechanisms for the two enzymes catalyzing steps in glycogen debranching based on their potential membership in the α-amylase family.

See answer

Chapter Integration and Data Interpretation Problems

16. *Glycogen isolation 1.* The liver is a major storage site for glycogen. Purified from two samples of human liver, glycogen was either treated or not treated with α-amylase and subsequently analyzed by SDS-PAGE and Western blotting with the use of antibodies to glycogenin. The results are presented in the adjoining illustration.

(a) Why are no proteins visible in the lanes without amylase treatment?

(b) What is the effect of treating the samples with α-amylase? Explain the results.

(c) List other proteins that you might expect to be associated with glycogen. Why are other proteins not visible?

See answer

17. *Glycogen isolation 2.* The gene for glycogenin was transfected into a cell line that normally stores only small amounts of glycogen. The cells were then manipulated according to the following protocol, and glycogen was isolated and analyzed by SDS-PAGE and Western blotting using an antibody to glycogenin with and without α-amylase treatment. The results are presented in the adjoining illustration.

The protocol: Cells cultured in growth medium and 25 mM glucose (lane 1) were switched to medium containing no glucose for 24 hours (lane 2). Glucose-starved cells were re-fed with medium containing 25 mM glucose for 1 hour (lane 3) or 3 hours (lane 4). Samples (12 μg of protein) were either treated or not treated with α-amylase, as indicated, before being loaded on the gel.

(a) Why did the Western analysis produce a "smear"—that is, the high-molecular-weight staining in lane 1(-)?

(b) What is the significance of the decrease in high-molecular-weight staining in lane 2(-)?

(c) What is the significance of the difference between lanes 2(-) and 3(-)?

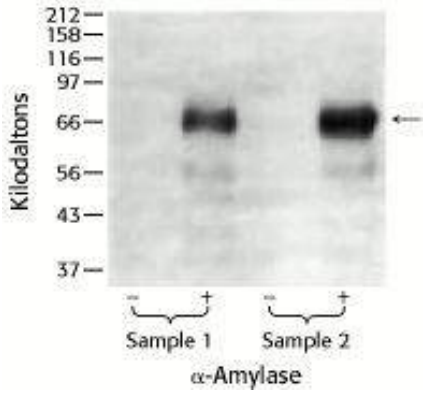
(d) Suggest a plausible reason why there is essentially no difference between lanes 3(-) and 4(-)?

(e) Why are the bands at 66 kd the same in the lanes treated with amylase, despite the fact that the cells were treated differently?

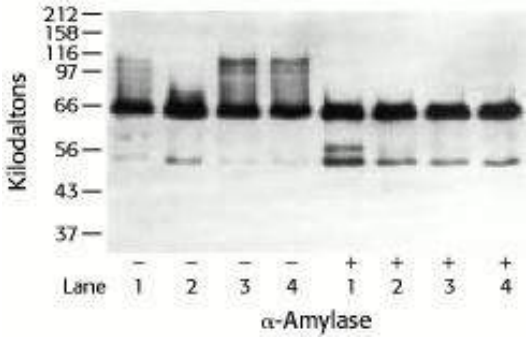
See answer

Media Problem

18. *Either/or.* Mammalian glycogen phosphorylase is activated by phosphorylation of serine 14, and deactivated by dephosphorylation. The binding of glucose to glycogen phosphorylase *a* inhibits the enzyme by promoting the protein phosphatase-catalyzed dephosphorylation of serine 14, which converts the *a* form of the enzyme to the *b* form. From the information presented in the **Structural Insights** module on glycogen phosphorylase, provide an explanation how glucose binding might promote dephosphorylation of serine 14.



Glycogen Isolation 1. [Courtesy of Dr. Peter J. Roach, Indiana University School of Medicine.]



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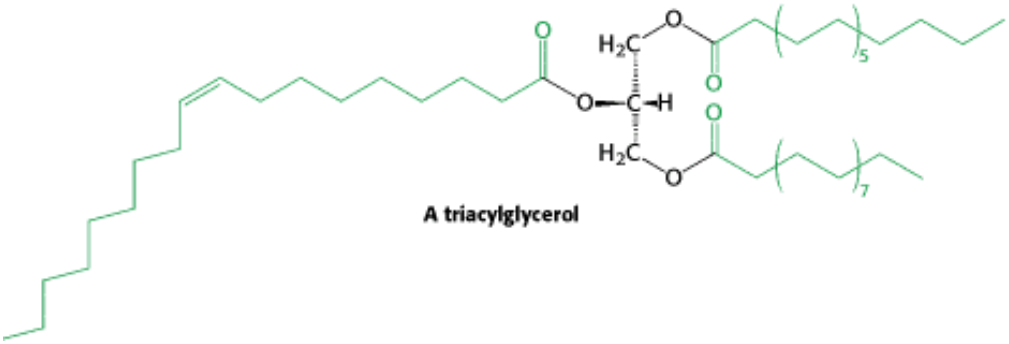
22. Fatty Acid Metabolism

We turn now from the metabolism of carbohydrates to that of fatty acids. A fatty acid contains a long hydrocarbon chain and a terminal carboxylate group. Fatty acids have four major physiological roles. First, *fatty acids are building blocks of phospholipids and glycolipids*. These amphipathic molecules are important components of biological membranes, as we discussed in Chapter 12. Second, many proteins are modified by the *covalent attachment of fatty acids, which targets them to membrane locations* (Section 12.5.3). Third, *fatty acids are fuel molecules*. They are stored as *triacylglycerols* (also called *neutral fats* or *triglycerides*), which are uncharged esters of fatty acids with glycerol (Figure 22.1). Fatty acids mobilized from triacylglycerols are oxidized to meet the energy needs of a cell or organism. Fourth, *fatty acid derivatives serve as hormones and intracellular messengers*. In this chapter, we will focus on the oxidation and synthesis of fatty acids, processes that are reciprocally regulated in response to hormones.

22.0.1. An Overview of Fatty Acid Metabolism

Fatty acid degradation and synthesis are relatively simple processes that are essentially the reverse of each other. The process of degradation converts an aliphatic compound into a set of activated acetyl units (acetyl CoA) that can be processed by the citric acid cycle (Figure 22.2). An activated fatty acid is oxidized to introduce a double bond; the double bond is hydrated to introduce an oxygen; the alcohol is oxidized to a ketone; and, finally, the four carbon fragment is cleaved by coenzyme A to yield acetyl CoA and a fatty acid chain two carbons shorter. If the fatty acid has an even number of carbon atoms and is saturated, the process is simply repeated until the fatty acid is completely converted into acetyl CoA units.



Fatty acid synthesis is essentially the reverse of this process. Because the result is a polymer, the process starts with monomers—in this case with activated acyl group (most simply, an acetyl unit) and malonyl units (see Figure 22.2). The malonyl unit is condensed with the acetyl unit to form a four-carbon fragment. To produce the required hydrocarbon chain, the carbonyl must be reduced. The fragment is reduced, dehydrated, and reduced again, exactly the opposite of degradation, to bring the carbonyl group to the level of a methylene group with the formation of butyryl CoA. Another activated malonyl group condenses with the butyryl unit and the process is repeated until a C_{16} fatty acid is synthesized.

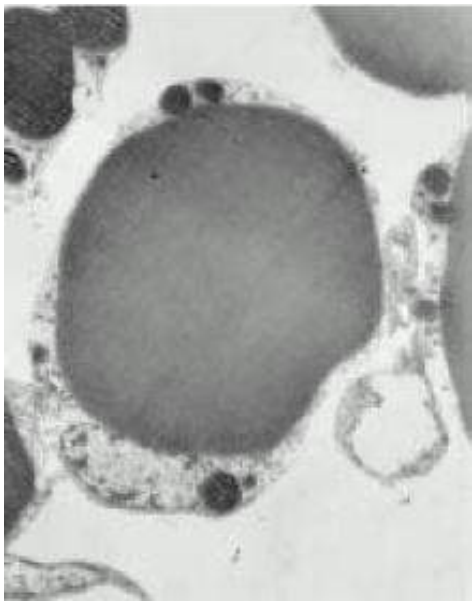


Figure 22.1. Electron Micrograph of an Adipocyte. A small band of cytoplasm surrounds the large deposit of triacylglycerols. [Biophoto Associates/ Photo Researchers.]

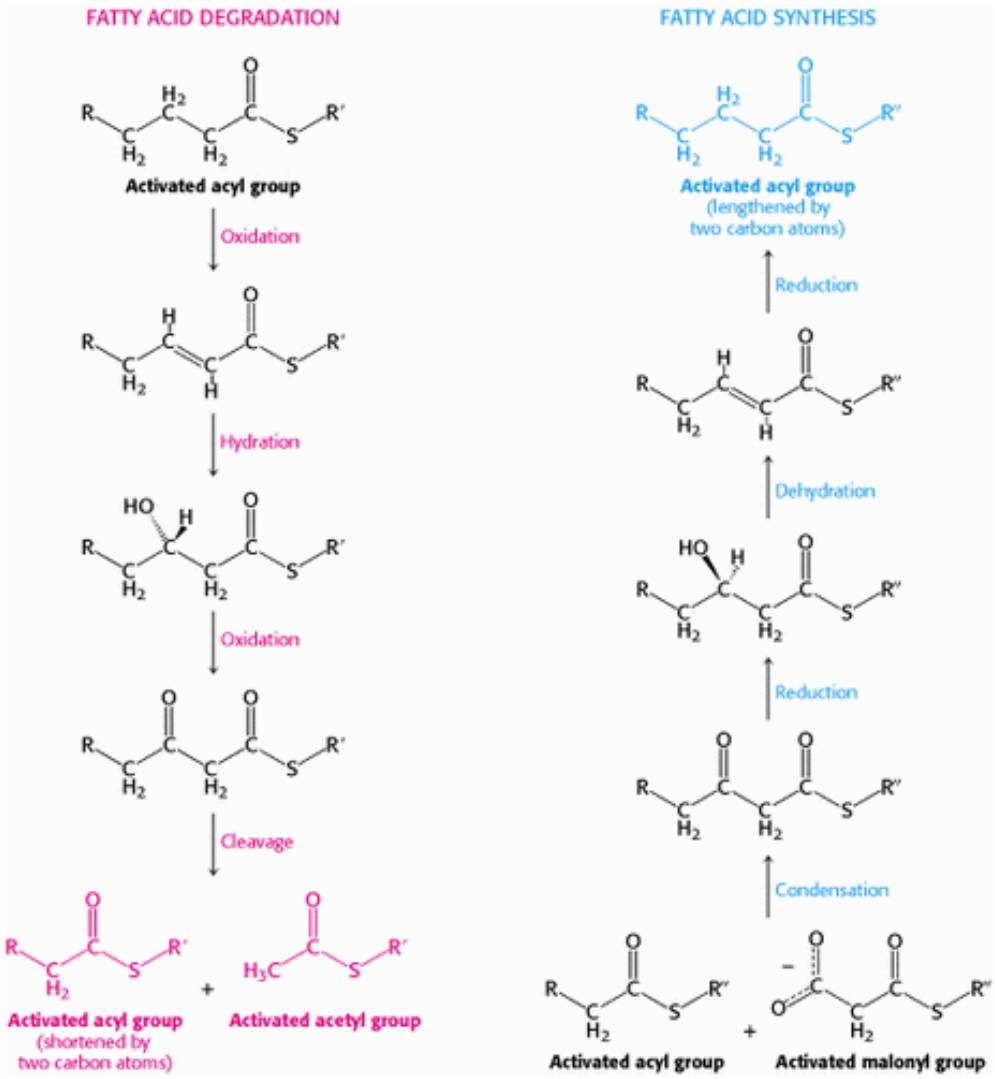
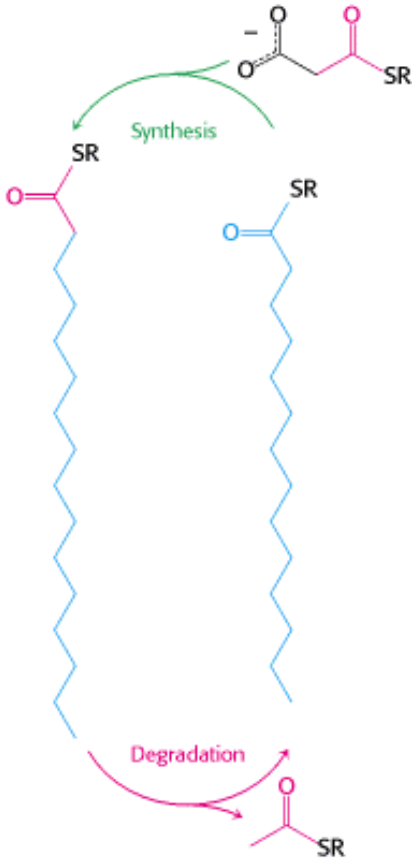


Figure 22.2. Steps in Fatty Acid Degradation and Synthesis. The two processes are in many ways mirror images of each other.



Fats provide an efficient means for storing energy for later use. (Right) The processes of fatty acid synthesis (preparation for energy storage) and fatty acid degradation (preparation for energy use) are, in many ways, the reverse of each other. (Above) Studies of mice are revealing the interplay between these pathways and the biochemical bases of appetite and weight control. [(Above) © Jackson/Visuals Unlimited.]

22.1. Triacylglycerols Are Highly Concentrated Energy Stores

Triacylglycerols are highly concentrated stores of metabolic energy because they are *reduced* and *anhydrous*. The yield from the complete oxidation of fatty acids is about 9 kcal g^{-1} (38 kJ g^{-1}), in contrast with about 4 kcal g^{-1} (17 kJ g^{-1}) for carbohydrates and proteins. The basis of this large difference in caloric yield is that fatty acids are much more reduced. Furthermore, triacylglycerols are nonpolar, and so they are stored in a nearly anhydrous form, whereas much more polar proteins and carbohydrates are more highly hydrated. In fact, 1 g of dry glycogen binds about 2 g of water. Consequently, *a gram of nearly anhydrous fat stores more than six times as much energy as a gram of hydrated glycogen*, which is likely the reason that triacylglycerols rather than glycogen were selected in evolution as the major energy reservoir. Consider a typical 70-kg man, who has fuel reserves of 100,000 kcal (420,000 kJ) in triacylglycerols, 25,000 kcal (100,000 kJ) in protein (mostly in muscle), 600 kcal (2500 kJ) in glycogen, and 40 kcal (170 kJ) in glucose. Triacylglycerols constitute about 11 kg of his total body weight. If this amount of energy were stored in glycogen, his total body weight would be 55 kg greater. The glycogen and glucose stores provide enough energy to sustain biological function for about 24 hours, whereas the triacylglycerol stores allow survival for several weeks.

In mammals, the major site of accumulation of triacylglycerols is the cytoplasm of *adipose cells (fat cells)*. Droplets of triacylglycerol coalesce to form a large globule, which may occupy most of the cell volume (see Figure 22.1). Adipose cells are specialized for the synthesis and storage of triacylglycerols and for their mobilization into fuel molecules that are transported to other tissues by the blood.

The utility of triacylglycerols as an energy source is dramatically illustrated by the abilities of migratory birds, which can fly great distances without eating. Examples are the American golden plover and the ruby-throated sparrow. The golden plover flies from Alaska to the southern tip of South America; a large segment of the flight (3800 km, or 2400 miles) is over open ocean, where the birds cannot feed. The ruby-throated hummingbird can fly nonstop across the Gulf of Mexico. Fatty acids provide the energy source for both these prodigious feats.

22.1.1. Dietary Lipids Are Digested by Pancreatic Lipases

Most lipids are ingested in the form of triacylglycerols but must be degraded to fatty acids for absorption across the intestinal epithelium. Recall that lipids are not easily solubilized, yet they must be in order to be degraded. Triacylglycerols in the intestinal lumen are incorporated into micelles formed with the aid of *bile salts* (Figure 22.3), amphipathic molecules synthesized from cholesterol in the liver and secreted from the gall bladder. Incorporation of lipids into micelles orients the ester bonds of the lipid toward the surface of the micelle, rendering the bonds more susceptible to digestion by pancreatic lipases that are in aqueous solution. If the production of bile salts is inadequate owing to liver disease, large amounts of fats (as much as 30 g day^{-1}) are excreted in the feces. This condition is referred to as steatorrhea, from the Greek *steato*, "fat."

The lipases digest the triacylglycerols into free fatty acids and monoacylglycerol (Figure 22.4). These digestion products are carried in micelles to the intestinal epithelium where they are absorbed across the plasma membrane.

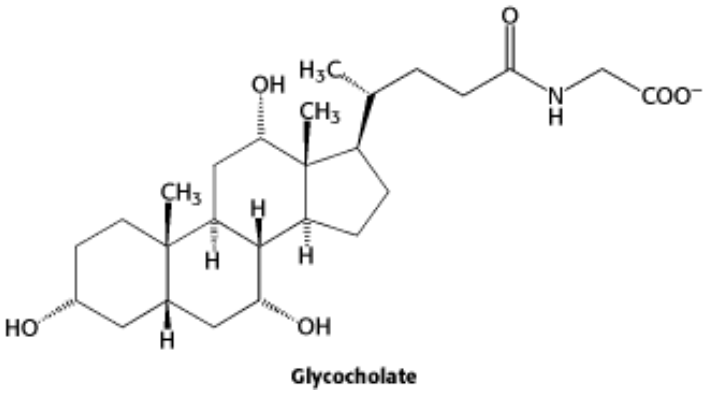
22.1.2. Dietary Lipids Are Transported in Chylomicrons

In the intestinal mucosal cells, the triacylglycerols are resynthesized from fatty acids and monoacylglycerols and then packaged into lipoprotein transport particles called *chylomicrons*, stable particles ranging from approximately 180 to 500 nm in diameter (Figure 22.5). These particles are composed mainly of triacylglycerols, with apoprotein B-48 as the main protein component. Protein constituents of lipoprotein particles are called *apolipoproteins*. Chylomicrons also function in the transport of fat-soluble vitamins and cholesterol.

The chylomicrons are released into the lymph system and then into the blood. These particles bind to membrane-bound lipoprotein lipases, primarily at adipose tissue and muscle, where the triacylglycerols are once again degraded into free

fatty acids and monoacylglycerol for transport into the tissue. The triacylglycerols are then resynthesized inside the cell and stored. In the muscle, they can be oxidized to provide energy, as will be discussed shortly.

Triacylglycerols fuel the long migration flights of the American Golden Plover (*Pluvialis dominica*). [Gerard Fuehrer/Visuals Unlimited.]



Glycocholate

Figure 22.3. Glycocholate. Bile salts, such as glycocholate, facilitate lipid digestion in the intestine.

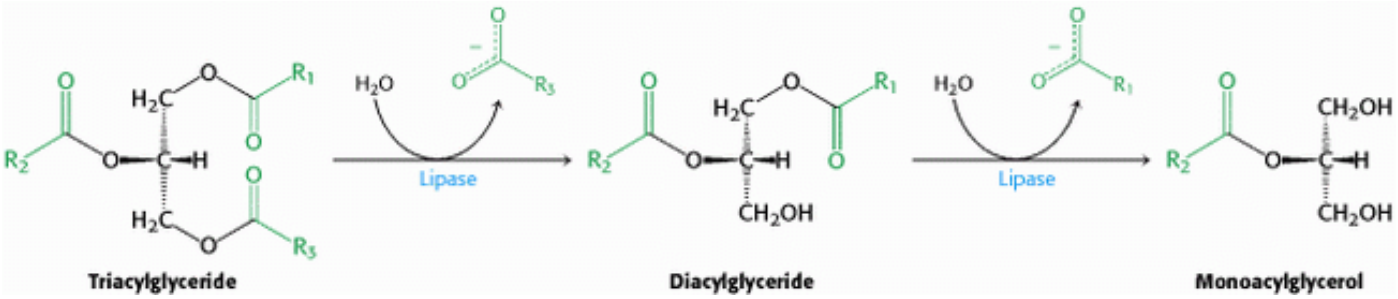


Figure 22.4. Action of Pancreatic Lipases. Lipases secreted by the pancreas convert triacylglycerols into fatty acids and monoacylglycerol for absorption into the intestine.

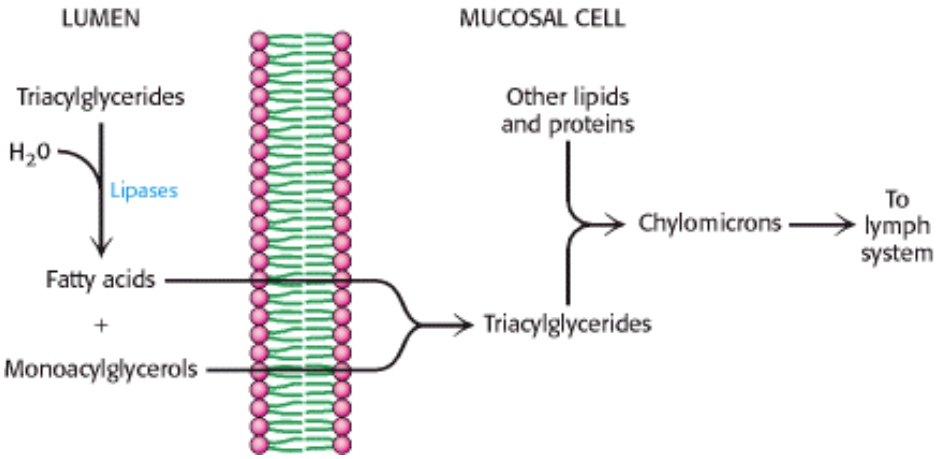
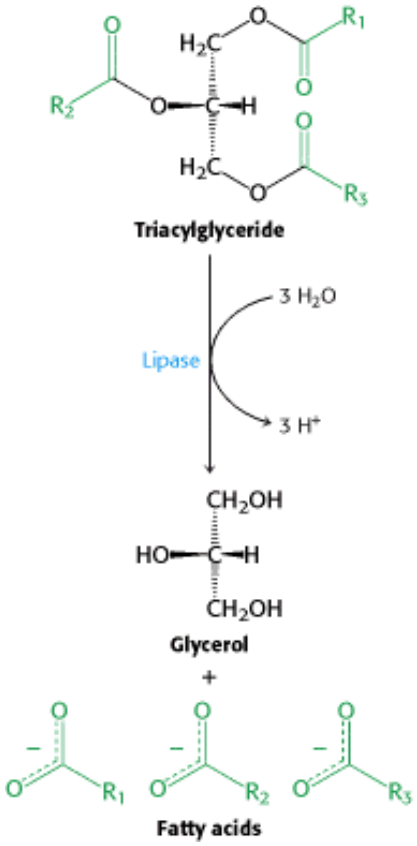


Figure 22.5. Chylomicron Formation. Free fatty acids and monoacylglycerols are absorbed by intestinal epithelial cells. Triacylglycerols are resynthesized and packaged with other lipids and apoprotein B-48 to form chylomicrons, which are then released into the lymph system.

22.2. The Utilization of Fatty Acids as Fuel Requires Three Stages of Processing

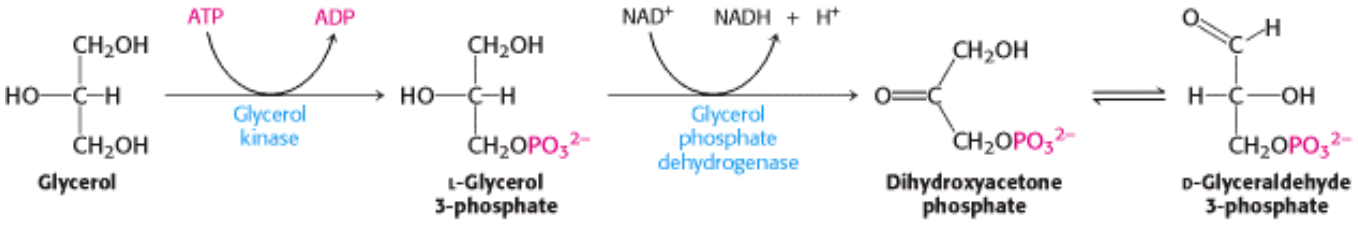
Peripheral tissues gain access to the lipid energy reserves stored in adipose tissue through three stages of processing. First, the lipids must be mobilized. In this process, triacylglycerols are degraded to fatty acids and glycerol, which are released from the adipose tissue and transported to the energy-requiring tissues. Second, at these tissues, the fatty acids must be activated and transported into mitochondria for degradation. Third, the fatty acids are broken down in a step-by-step fashion into acetyl CoA, which is then processed in the citric acid cycle.



22.2.1. Triacylglycerols Are Hydrolyzed by Cyclic AMP-Regulated Lipases

The initial event in the utilization of fat as an energy source is the hydrolysis of triacylglycerols by lipases, an event referred to as *lipolysis*. The lipase of adipose tissue are activated on treatment of these cells with the hormones epinephrine, norepinephrine, glucagon, and adrenocorticotropic hormone. In adipose cells, these hormones trigger 7TM receptors that activate adenylate cyclase (Section 15.1.3). The increased level of cyclic AMP then stimulates protein kinase A, which activates the lipases by phosphorylating them. Thus, *epinephrine, norepinephrine, glucagon, and adrenocorticotropic hormone induce lipolysis* (Figure 22.6). In contrast, *insulin inhibits lipolysis*. The released fatty acids are not soluble in blood plasma, and so, on release, serum albumin binds the fatty acids and serves as a carrier. By these means, free fatty acids are made accessible as a fuel in other tissues.

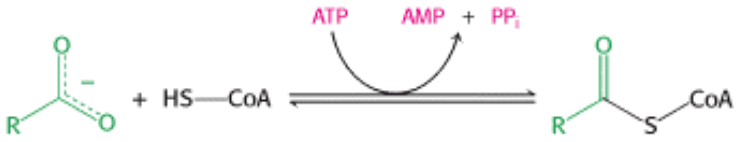
Glycerol formed by lipolysis is absorbed by the liver and phosphorylated, oxidized to dihydroxyacetone phosphate, and then isomerized to glyceraldehyde 3-phosphate. This molecule is an intermediate in both the glycolytic and the gluconeogenic pathways.



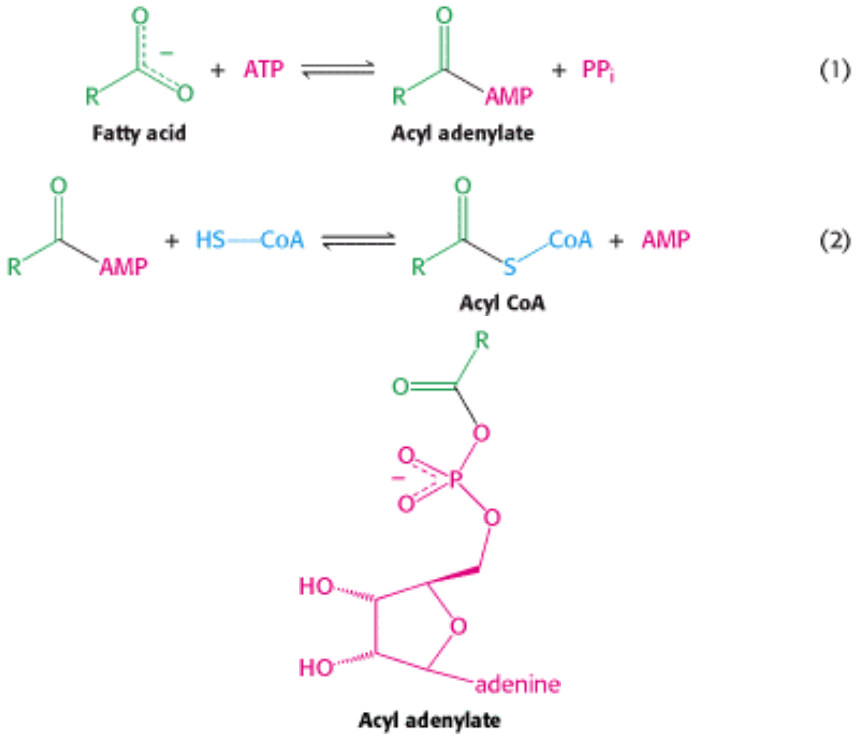
Hence, glycerol can be converted into pyruvate or glucose in the liver, which contains the appropriate enzymes. The reverse process can take place by the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate. Hydrolysis by a phosphatase then gives glycerol. Thus, glycerol and glycolytic intermediates are readily interconvertible.

22.2.2. Fatty Acids Are Linked to Coenzyme A Before They Are Oxidized

Eugene Kennedy and Albert Lehninger showed in 1949 that fatty acids are oxidized in mitochondria. Subsequent work demonstrated that they are activated before they enter the mitochondrial matrix. Adenosine triphosphate (ATP) drives the formation of a thioester linkage between the carboxyl group of a fatty acid and the sulfhydryl group of CoA. This activation reaction takes place on the outer mitochondrial membrane, where it is catalyzed by *acyl CoA synthetase* (also called *fatty acid thiokinase*).



Paul Berg showed that the activation of a fatty acid is accomplished in two steps. First, the fatty acid reacts with ATP to form an *acyl adenylate*. In this mixed anhydride, the carboxyl group of a fatty acid is bonded to the phosphoryl group of AMP. The other two phosphoryl groups of the ATP substrate are released as pyrophosphate. The sulfhydryl group of CoA then attacks the acyl adenylate, which is tightly bound to the enzyme, to form acyl CoA and AMP.



These partial reactions are freely reversible. In fact, the equilibrium constant for the sum of these reactions is close to 1. One high-transfer-potential compound is cleaved (between PP_i and AMP) and one high-transfer-potential compound is formed (the thioester acyl CoA). How is the overall reaction driven forward? The answer is that pyrophosphate is rapidly hydrolyzed by a pyrophosphatase, and so the complete reaction is

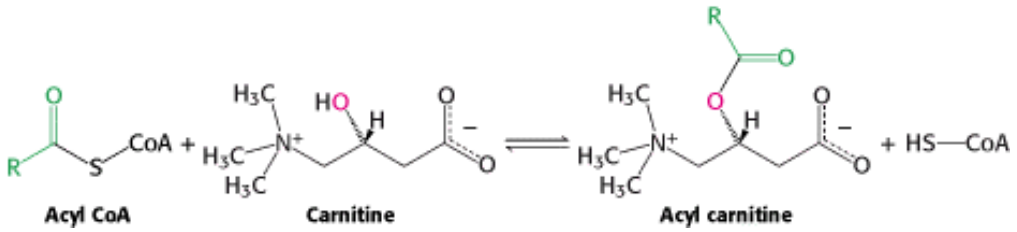
$$RCOO^- + CoA + ATP + H_2O \longrightarrow RCO{-}CoA + AMP + P_i + 2\,H^+$$

This reaction is quite favorable because the equivalent of two molecules of ATP is hydrolyzed, whereas only one high-transfer-potential compound is formed. We see here another example of a recurring theme in biochemistry: *many biosynthetic reactions are made irreversible by the hydrolysis of inorganic pyrophosphate*.

Another motif recurs in this activation reaction. The enzyme-bound acyl-adenylate intermediate is not unique to the synthesis of acyl CoA. *Acyl adenylates are frequently formed when carboxyl groups are activated in biochemical reactions*. Amino acids are activated for protein synthesis by a similar mechanism (Section 29.2.1), although the enzymes that catalyze this process are not homologous to acyl CoA synthetase. Thus, *activation by adenylation recurs in part because of convergent evolution*.

22.2.3. Carnitine Carries Long-Chain Activated Fatty Acids into the Mitochondrial Matrix

Fatty acids are activated on the outer mitochondrial membrane, whereas they are oxidized in the mitochondrial matrix. A special transport mechanism is needed to carry long-chain acyl CoA molecules across the inner mitochondrial membrane. Activated long-chain fatty acids are transported across the membrane by conjugating them to *carnitine*, a zwitterionic alcohol. The acyl group is transferred from the sulfur atom of CoA to the hydroxyl group of carnitine to form *acyl carnitine*. This reaction is catalyzed by *carnitine acyltransferase I* (also called *carnitine palmitoyl transferase I*), which is bound to the outer mitochondrial membrane.



Acyl carnitine is then shuttled across the inner mitochondrial membrane by a translocase (Figure 22.7). The acyl group is transferred back to CoA on the matrix side of the membrane. This reaction, which is catalyzed by *carnitine acyltransferase II (carnitine palmitoyl transferase II)*, is simply the reverse of the reaction that takes place in the cytosol. Normally, the transfer of an acyl group from an alcohol to a sulfhydryl group is thermodynamically unfavorable. However, the equilibrium constant for this reaction for carnitine is near 1, apparently because carnatine and its esters are solvated differently from most other alcohols and their esters because of the zwitterionic nature of carnitine. As a result, the *O*-acyl link in carnitine has a high group-transfer potential. Finally, the translocase returns carnitine to the cytosolic side in exchange for an incoming acyl carnitine.

A number of diseases have been traced to a deficiency of carnitine, the transferase or the translocase. The symptoms of carnitine deficiency range from mild muscle cramping to severe weakness and even death. The muscle, kidney, and heart are the tissues primarily affected. Muscle weakness during prolonged exercise is an important characteristic of a deficiency of carnitine acyl transferases because muscle relies on fatty acids as a long-term source of energy. Medium-chain (C_8-C_{10}) fatty acids, which do not require carnitine to enter the mitochondria, are oxidized normally in these patients. These diseases illustrate that the impaired flow of a metabolite from one compartment of a cell to another can lead to a pathological condition.

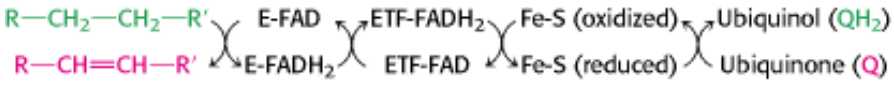
22.2.4. Acetyl CoA, NADH, and $FADH_2$ Are Generated in Each Round of Fatty Acid Oxidation

A saturated acyl CoA is degraded by a recurring sequence of four reactions: oxidation by flavin adenine dinucleotide (FAD), hydration, oxidation by NAD^+, and thiolysis by CoA (Figure 22.8). The fatty acyl chain is shortened by two carbon atoms as a result of these reactions, and $FADH_2$, NADH, and acetyl CoA are generated. Because oxidation is on the β carbon, this series of reactions is called the β-*oxidation pathway*.

The first reaction in each round of degradation is the *oxidation* of acyl CoA by an *acyl CoA dehydrogenase* to give an enoyl CoA with a trans double bond between C-2 and C-3.

$$\text{Acyl CoA} + \text{E-FAD} \longrightarrow \textit{trans}\text{-}\Delta^2\text{-enoyl CoA} + \text{E-FADH}_2$$

As in the dehydrogenation of succinate in the citric acid cycle, FAD rather than NAD^+ is the electron acceptor because the value of ΔG for this reaction is insufficient to drive the reduction of NAD^+. Electrons from the $FADH_2$ prosthetic group of the reduced acyl CoA dehydrogenase are transferred to a second flavoprotein called *electron-transferring flavoprotein* (ETF). In turn, ETF donates electrons to *ETF:ubiquinone reductase*, an iron-sulfur protein. Ubiquinone is thereby reduced to ubiquinol, which delivers its high-potential electrons to the second proton-pumping site of the respiratory chain (Section 18.3.3). Consequently, 1.5 molecules of ATP are generated per molecule of $FADH_2$ formed in this dehydrogenation step, as in the oxidation of succinate to fumarate (Section 18.3.2).



The next step is the *hydration* of the double bond between C-2 and C-3 by *enoyl CoA hydratase*.

$$\textit{trans}\text{-}\Delta^2\text{-Enoyl CoA} + H_2O \rightleftharpoons \text{L-3-hydroxyacyl CoA}$$

The hydration of enoyl CoA is stereospecific. Only the l isomer of 3-hydroxyacyl CoA is formed when the trans-Δ^2 double bond is hydrated. The enzyme also hydrates a cis-Δ^2 double bond, but the product then is the d isomer. We shall return to this point shortly in considering how unsaturated fatty acids are oxidized.

The hydration of enoyl CoA is a prelude to the second *oxidation* reaction, which converts the hydroxyl group at C-3 into a keto group and generates NADH. This oxidation is catalyzed by *l-3-hydroxyacyl CoA dehydrogenase*, which is specific for the l isomer of the hydroxyacyl substrate.

$$\text{L-3-Hydroxyacyl CoA} + NAD^+ \rightleftharpoons \text{3-ketoacyl CoA} + NADH + H^+$$

The preceding reactions have oxidized the methylene group at C-3 to a keto group. The final step is the cleavage of 3-ketoacyl CoA by the thiol group of a second molecule of CoA, which yields acetyl CoA and an acyl CoA shortened by two carbon atoms. This thiolytic cleavage is catalyzed by β-*ketothiolase*.

$$\underset{(n \text{ carbons})}{\text{3-Ketoacyl CoA}} + \text{HS—CoA} \rightleftharpoons \text{acetyl CoA} + \underset{(n-2 \text{ carbons})}{\text{acyl CoA}}$$

Table 22.1 summarizes the reactions in fatty acid oxidation.

The shortened acyl CoA then undergoes another cycle of oxidation, starting with the reaction catalyzed by acyl CoA dehydrogenase (Figure 22.9). Fatty acyl chains containing from 12 to 18 carbon atoms are oxidized by the long-chain acyl CoA dehydrogenase. The medium-chain acyl CoA dehydrogenase oxidizes fatty acyl chains having from 14 to 4 carbons, whereas the short-chain acyl CoA dehydrogenase acts only on 4- and 6- carbon acyl chains. In contrast, β-ketothiolase, hydroxyacyl dehydrogenase, and enoyl CoA hydratase have broad specificity with respect to the length of the acyl group.

22.2.5. The Complete Oxidation of Palmitate Yields 106 Molecules of ATP

We can now calculate the energy yield derived from the oxidation of a fatty acid. In each reaction cycle, an acyl CoA is shortened by two carbon atoms, and one molecule each of $FADH_2$, NADH, and acetyl CoA is formed.

$$\begin{aligned} &C_n\text{-acyl CoA} + FAD + NAD^+ + H_2O + CoA \longrightarrow \\ &\qquad C_{n-2}\text{-acyl CoA} + FADH_2 + NADH + \text{acetyl CoA} + H^+ \end{aligned}$$

The degradation of palmitoyl CoA (C_{16}-acyl CoA) requires seven reaction cycles. In the seventh cycle, the C_4-ketoacyl CoA is thiolyzed to two molecules of acetyl CoA. Hence, the stoichiometry of oxidation of palmitoyl CoA is

$$\begin{aligned} &\text{Palmitoyl CoA} + 7\,FAD + 7\,NAD^+ + 7\,CoA + 7\,H_2O \longrightarrow \\ &\qquad 8\,\text{acetyl CoA} + 7\,FADH_2 + 7\,NADH + 7\,H^+ \end{aligned}$$

Approximately 2.5 molecules of ATP are generated when the respiratory chain oxidizes each of the 7 molecules of NADH, whereas 1.5 molecules of ATP are formed for each of the 7 molecules of $FADH_2$ because their electrons enter the chain at the level of ubiquinol. Recall that the oxidation of acetyl CoA by the citric acid cycle yields 10 molecules of ATP. Hence, the number of ATP molecules formed in the oxidation of palmitoyl CoA is 10.5 from the 7 molecules of $FADH_2$, 17.5 from the 7 molecules of NADH, and 80 from the 8 molecules of acetyl CoA, which gives a total of 108. The equivalent of 2 molecules of ATP is consumed in the activation of palmitate, in which ATP is split into AMP and 2 molecules of P_i. Thus, *the complete oxidation of a molecule of palmitate yields 106 molecules of ATP*.

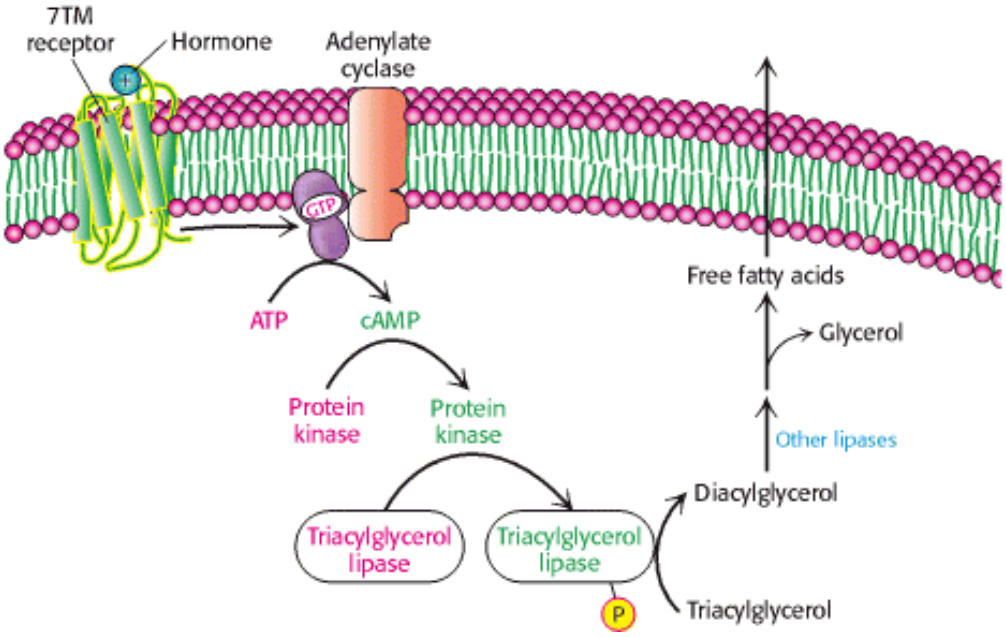


Figure 22.6. Mobilization of Triacylglycerols. Triacylglycerols in adipose tissue are converted into free fatty acids and glycerol for release into the bloodstream in response to hormonal signals. A hormone-sensitive lipase initiates the process.

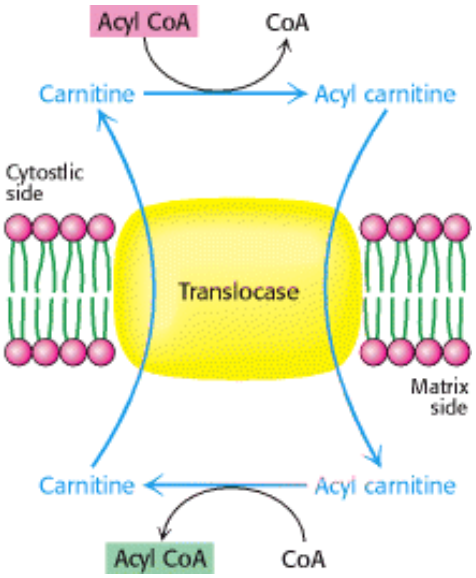


Figure 22.7. Acyl Carnitine Translocase. The entry of acyl carnitine into the mitochondrial matrix is mediated by a translocase. Carnitine returns to the cytosolic side of the inner mitochondrial membrane in exchange for acyl carnitine.

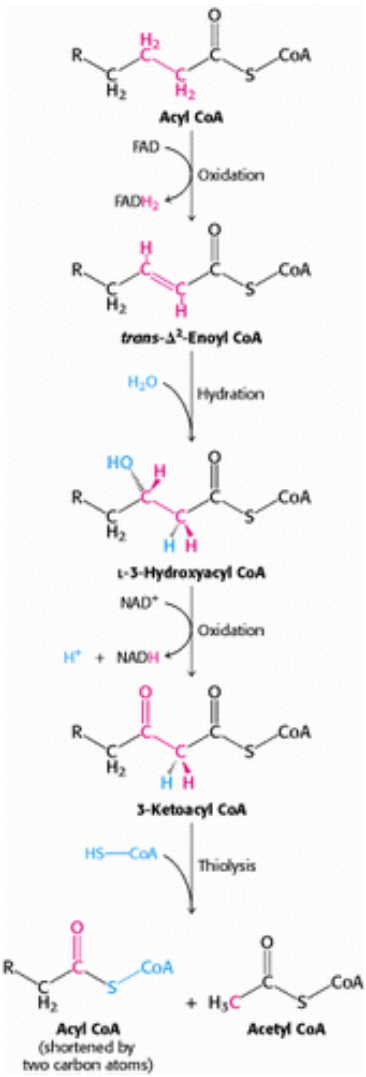


Figure 22.8. Reaction Sequence for the Degradation of Fatty Acids. Fatty acids are degraded by the repetition of a

four-reaction sequence consisting of oxidation, hydration, oxidation, and thiolysis.

Table 22.1. Principal reactions in fatty acid oxidation

Step	Reaction	Enzyme
1	Fatty acid + CoA + ATP $\rightleftharpoons$ acyl CoA + AMP + PP_i	Acyl CoA synthetase [also called fatty acid thiokinase and fatty acid:CoA ligase (AMP)]
2	Carnitine + acyl CoA $\rightleftharpoons$ acyl carnitine + CoA	Carnitine acyltransferase (also called carnitine palmitoyl transferase)
3	Acyl CoA + E-FAD $\rightarrow$ *trans*-Δ^2-enoyl CoA + E-$FADH_2$	Acyl CoA dehydrogenases (several isozymes having different chain-length specificity)
4	*trans*-Δ^2-Enoyl CoA + H_2O $\rightleftharpoons$ l-3-hydroxyacyl CoA	Enoyl CoA hydratase (also called crotonase or 3-hydroxyacyl CoA hydrolyase)
5	l-3-Hydroxyacyl CoA + NAD^+ $\rightleftharpoons$ 3-ketoacyl CoA + NADH + H^+	l-3-Hydroxyacyl CoA dehydrogenase
6	3-ketoacyl CoA + CoA $\rightleftharpoons$ acetyl CoA + acyl CoA (shortened by C_2)	β-Ketothiolase (also called thiolase)

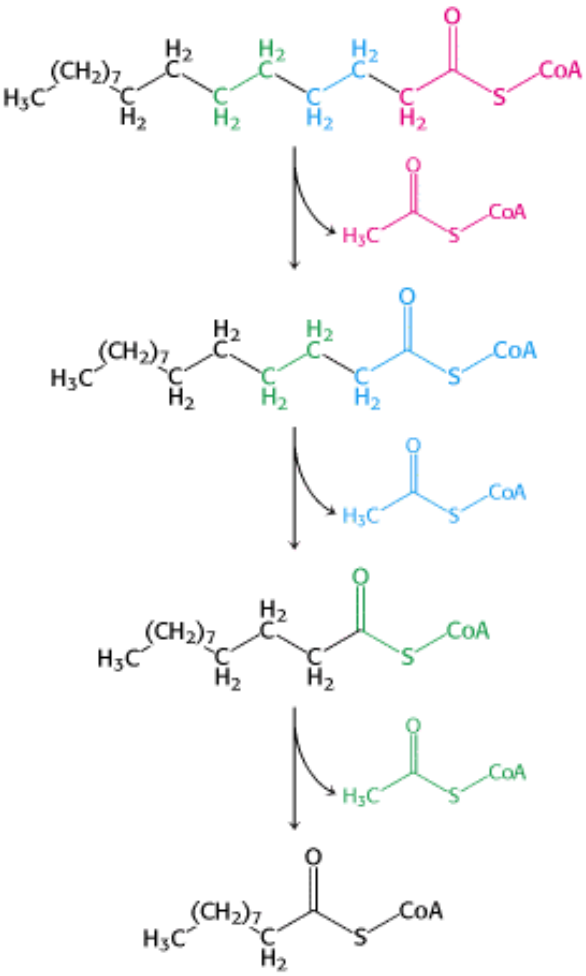


Figure 22.9. First Three Rounds in the Degradation of Palmitate. Two-carbon units are sequentially removed from the carboxyl end of the fatty acid.

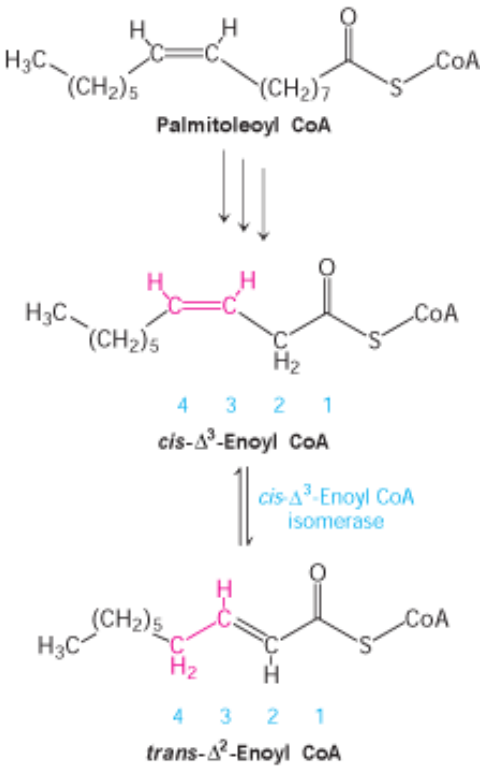
22.3. Certain Fatty Acids Require Additional Steps for Degradation

The β-oxidation pathway accomplishes the complete degradation of saturated fatty acids having an even number of carbon atoms. Most fatty acids have such structures because of their mode of synthesis (Section 22.4.3). However, not all fatty acids are so simple. The oxidation of fatty acids containing double bonds requires additional steps. Likewise, fatty acids containing an odd number of carbon atoms yield a propionyl CoA at the final thiolysis step that must be converted into an easily usable form by additional enzyme reactions.

22.3.1. An Isomerase and a Reductase Are Required for the Oxidation of Unsaturated Fatty Acids

The oxidation of unsaturated fatty acids presents some difficulties, yet many such fatty acids are available in the diet. Most of the reactions are the same as those for saturated fatty acids. In fact, only two additional enzymes—an isomerase and a reductase—are needed to degrade a wide range of unsaturated fatty acids.

Consider the oxidation of palmitoleate. This C_{16} unsaturated fatty acid, which has one double bond between C-9 and C-10, is activated and transported across the inner mitochondrial membrane in the same way as saturated fatty acids. Palmitoleoyl CoA then undergoes three cycles of degradation, which are carried out by the same enzymes as in the oxidation of saturated fatty acids. However, the *cis*-Δ^3-enoyl CoA formed in the third round is not a substrate for acyl CoA dehydrogenase. The presence of a double bond between C-3 and C-4 prevents the formation of another double bond between C-2 and C-3. This impasse is resolved by a new reaction that shifts the position and configuration of the cis-Δ^3 double bond. *An isomerase converts this double bond into a trans-* Δ^2 *double bond*. The subsequent reactions are those of the saturated fatty acid oxidation pathway, in which the *trans*-Δ^2-enoyl CoA is a regular substrate.

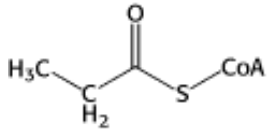


Another problem arises with the oxidation of polyunsaturated fatty acids. Consider linoleate, a C_{18} polyunsaturated fatty

acid with cis-Δ^9 and cis-Δ^{12} double bonds (Figure 22.10). The cis-Δ^3 double bond formed after three rounds of β oxidation is converted into a trans-Δ^2 double bond by the aforementioned isomerase. The acyl CoA produced by another round of β oxidation contains a cis-Δ^4 double bond. Dehydrogenation of this species by acyl CoA dehydrogenase yields a *2,4-dienoyl intermediate*, which is not a substrate for the next enzyme in the β-oxidation pathway. This impasse is circumvented by *2,4-dienoyl CoA reductase*, an enzyme that uses NADPH to reduce the 2,4-dienoyl intermediate to *trans*-Δ^3-enoyl CoA. *cis*- Δ^3-Enoyl CoA isomerase then converts *trans*-Δ^3-enoyl CoA into the trans-Δ^2 form, a customary intermediate in the β-oxidation pathway. These catalytic strategies are elegant and economical. Only two extra enzymes are needed for the oxidation of *any* polyunsaturated fatty acid. *Odd-numbered double bonds are handled by the isomerase, and even-numbered ones by the reductase and the isomerase*.

22.3.2. Odd-Chain Fatty Acids Yield Propionyl Coenzyme A in the Final Thiolysis Step

Fatty acids having an odd number of carbon atoms are minor species. They are oxidized in the same way as fatty acids having an even number, except that propionyl CoA and acetyl CoA, rather than two molecules of acetyl CoA, are produced in the final round of degradation. The activated three-carbon unit in propionyl CoA enters the citric acid cycle after it has been converted into succinyl CoA.



Propionyl CoA

22.3.3. Propionyl CoA Is Converted into Succinyl CoA in a Reaction That Requires Vitamin B_{12}

The pathway from propionyl CoA to succinyl CoA is especially interesting because it entails a rearrangement that requires *vitamin* B_{12} (also known as *cobalamin*). Propionyl CoA is carboxylated at the expense of the hydrolysis of an ATP to yield the d isomer of methylmalonyl CoA (Figure 22.11). This carboxylation reaction is catalyzed by *propionyl CoA carboxylase*, a biotin enzyme that is homologous to and has a catalytic mechanism like that of pyruvate carboxylase (Section 16.3.2). The d isomer of methylmalonyl CoA is racemized to the l isomer, the substrate for a mutase that converts it into *succinyl CoA* by an *intramolecular rearrangement*. The -CO-S-CoA group migrates from C-2 to C-3 in exchange for a hydrogen atom. This very unusual isomerization is catalyzed by *methylmalonyl CoA mutase*, which contains a derivative of vitamin B_{12}, cobalamin, as its coenzyme.

Cobalamin enzymes, which are present in most organisms, catalyze three types of reactions: (1) *intramolecular rearrangements;* (2) *methylations*, as in the synthesis of methionine (Section 24.2.7); and (3) *reduction of ribonucleotides to deoxyribonucleotides* (Section 25.3). In mammals, the conversion of l-methylmalonyl CoA into succinyl CoA and the formation of methionine by methylation of homocysteine are the only reactions that are known to require coenzyme B_{12}. The latter reaction is especially important because methionine is required for the generation of coenzymes that participate in the synthesis of purines and thymine, which are needed for nucleic acid synthesis.

The core of cobalamin consists of a *corrin ring with a central cobalt atom* (Figure 22.12). The corrin ring, like a porphyrin, has *four pyrrole units*. Two of them are directly bonded to each other, whereas methene bridges, as in porphyrins, join the others. The corrin ring is more reduced than that of porphyrins and the substituents are different. A cobalt atom is bonded to the four pyrrole nitrogens. Linked to the corrin ring is a derivative of *di-methylbenzimidazole* that contains ribose 3-phosphate and aminoisopropanol. In free cobalamin, one of the nitrogen atoms of dimethylbenzimidazole is the *fifth substituent* linked to the cobalt atom. In coenzyme B_{12}, *the sixth substituent* linked to the cobalt atom is a $5'$*-deoxyadenosyl unit*. This position can also be occupied by a cyano group, a methyl group, or

other ligands. In these compounds, the cobalt is in the +3 oxidation state.

The rearrangement reactions catalyzed by coenzyme B_{12} are exchanges of two groups attached to adjacent carbon atoms (Figure 22.13). A hydrogen atom migrates from one carbon atom to the next, and an R group (such as the -CO-S-CoA group of methylmalonyl CoA) concomitantly moves in the reverse direction. The first step in these intramolecular rearrangements is the cleavage of the carbon-cobalt bond of 5′-deoxyadenosylcobalamin to form coenzyme B_{12} (Co^{2+}) and a 5′-deoxyadenosyl radical, $-CH_2\cdot$) (Figure 22.14). In this *homolytic cleavage reaction*, one electron of the Co-C bond stays with Co (reducing it from the +3 to the +2 oxidation state) while the other stays with the carbon atom, generating a free radical. In contrast, nearly all other cleavage reactions in biological systems are *heterolytic* —an electron *pair* is transferred to one of the two atoms that were bonded together.

What is the role of this very unusual $-CH_2\cdot$ radical? This highly reactive species abstracts a *hydrogen atom* from the substrate to form 5′-deoxyadenosine and a substrate radical (Figure 22.15). This substrate radical spontaneously rearranges: the carbonyl CoA group migrates to the position formerly occupied by H on the neighboring carbon atom to produce a different radical. This product radical abstracts a hydrogen atom from the methyl group of 5′-deoxyadenosine to complete the rearrangement and return the deoxyadenosyl unit to the radical form. *The role of coenzyme* B_{12} *in such intramolecular migrations is to serve as a source of free radicals for the abstraction of hydrogen atoms*.

An essential property of coenzyme B_{12} is the weakness of its cobalt-carbon bond, the facile cleavage of which generates a radical. To facilitate the cleavage of this bond, enzymes such as methylmalonyl CoA mutase displace the benzamidazole group from the cobalt and coordinate the cobalamin through a histidine residue (Figure 22.16). The steric crowding around the cobalt-carbon bond within the corrin ring system contributes to the bond weakness.

22.3.4. Fatty Acids Are Also Oxidized in Peroxisomes

Although most fatty acid oxidation takes place in mitochondria, some oxidation takes place in cellular organelles called *peroxisomes* (Figure 22.17). These organelles are characterized by high concentrations of the enzyme catalase, which catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen (Section 18.3.6). Fatty acid oxidation in these organelles, which halts at octanyl CoA, may serve to shorten long chains to make them better substrates of β oxidation in mitochondria. Peroxisomal oxidation differs from β oxidation in the initial dehydrogenation reaction (Figure 22.18). In peroxisomes, a flavoprotein dehydrogenase transfers electrons to O_2 to yield H_2O_2 instead of capturing the high-energy electrons as $FADH_2$, as occurs in mitochondrial β oxidation. Catalase is needed to convert the hydrogen peroxide produced in the initial reaction into water and oxygen. Subsequent steps are identical with their mitochondrial counterparts, although they are carried out by different isoforms of the enzymes.


Zellweger syndrome, which results from the absence of functional peroxisomes, is characterized by liver, kidney, and muscle abnormalities and usually results in death by age six. The syndrome is caused by a defect in the import of enzymes into the peroxisomes. Here we see a pathological condition resulting from an inappropriate cellular distribution of enzymes.

22.3.5. Ketone Bodies Are Formed from Acetyl Coenzyme A When Fat Breakdown Predominates

The acetyl CoA formed in fatty acid oxidation enters the citric acid cycle only if fat and carbohydrate degradation are appropriately balanced. The reason is that the entry of acetyl CoA into the citric acid cycle depends on the availability of oxaloacetate for the formation of citrate, but the concentration of oxaloacetate is lowered if carbohydrate is unavailable or improperly utilized. Recall that oxaloacetate is normally formed from pyruvate, the product of glycolysis, by pyruvate carboxylase (Section 16.3.1). This is the molecular basis of the adage that *fats burn in the flame of carbohydrates*.

In fasting or diabetes, oxaloacetate is consumed to form glucose by the gluconeogenic pathway (Section 16.3.2) and hence is unavailable for condensation with acetyl CoA. Under these conditions, acetyl CoA is diverted to the formation of acetoacetate and D-3-hydroxybutyrate. Acetoacetate, D-3-hydroxybutyrate, and acetone are often referred to as *ketone bodies*. Abnormally high levels of ketone bodies are present in the blood of untreated diabetics (Section 22.3.6).

Acetoacetate is formed from acetyl CoA in three steps (Figure 22.19). Two molecules of acetyl CoA condense to form acetoacetyl CoA. This reaction, which is catalyzed by thiolase, is the reverse of the thiolysis step in the oxidation of fatty acids. Acetoacetyl CoA then reacts with acetyl CoA and water to give 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) and CoA. This condensation resembles the one catalyzed by citrate synthase (Section 17.1.3). This reaction, which has a favorable equilibrium owing to the hydrolysis of a thioester linkage, compensates for the unfavorable equilibrium in the formation of acetoacetyl CoA. 3-Hydroxy-3-methylglutaryl CoA is then cleaved to acetyl CoA and acetoacetate. The sum of these reactions is

$$2\ \text{Acetyl CoA} + H_2O \longrightarrow \text{acetoacetate} + 2\ \text{CoA} + H^+$$

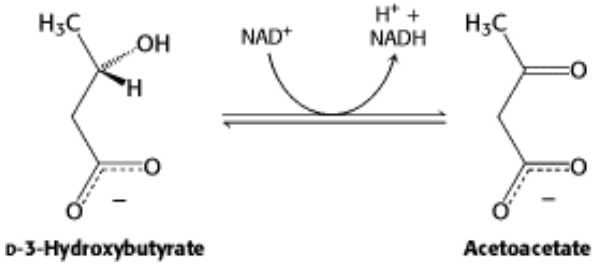
D-3-Hydroxybutyrate is formed by the reduction of acetoacetate in the mitochondrial matrix by D-3-hydroxybutyrate dehydrogenase. The ratio of hydroxybutyrate to acetoacetate depends on the NADH/NAD^+ ratio inside mitochondria.

Because it is a β-ketoacid, acetoacetate also undergoes a slow, spontaneous decarboxylation to acetone. The odor of acetone may be detected in the breath of a person who has a high level of acetoacetate in the blood.

22.3.6. Ketone Bodies Are a Major Fuel in Some Tissues

The major site of production of acetoacetate and 3-hydroxybutyrate is the liver. These substances diffuse from the liver mitochondria into the blood and are transported to peripheral tissues. These ketone bodies were initially regarded as degradation products of little physiological value. However, the results of studies by George Cahill and others revealed that these derivatives of acetyl CoA are important molecules in energy metabolism. *Acetoacetate and 3-hydroxybutyrate are normal fuels of respiration and are quantitatively important as sources of energy*. Indeed, heart muscle and the renal cortex use acetoacetate in preference to glucose. In contrast, glucose is the major fuel for the brain and red blood cells in well-nourished people on a balanced diet. However, the brain adapts to the utilization of acetoacetate during starvation and diabetes (Sections 30.3.1 and 30.3.2). In prolonged starvation, 75% of the fuel needs of the brain are met by ketone bodies.

3-Hydroxybutyrate is oxidized to produce acetoacetate as well as NADH for use in oxidative phosphorylation.



Acetoacetate can be activated by the transfer of CoA from succinyl CoA in a reaction catalyzed by a specific CoA transferase. Acetoacetyl CoA is then cleaved by thiolase to yield two molecules of acetyl CoA, which can then enter the citric acid cycle (Figure 22.20). The liver has acetoacetate available to supply to other organs because it lacks this particular CoA transferase.

Ketone bodies can be regarded as a water-soluble, transportable form of acetyl units. Fatty acids are released by adipose tissue and converted into acetyl units by the liver, which then exports them as acetoacetate. As might be expected, acetoacetate also has a regulatory role. *High levels of acetoacetate in the blood signify an abundance of acetyl units and*

lead to a decrease in the rate of lipolysis in adipose tissue.

Certain pathological conditions can lead to a life-threatening rise in the blood levels of the ketone bodies. Most common of these conditions is diabetic ketosis in patients with insulin-dependent diabetes mellitus. The absence of insulin has two major biochemical consequences. First, the liver cannot absorb glucose and consequently cannot provide oxaloacetate to process fatty acid-derived acetyl CoA (Section 17.3.1). Second, insulin normally curtails fatty acid mobilization by adipose tissue. The liver thus produces large amounts of ketone bodies, which are moderately strong acids. The result is severe acidosis. The decrease in pH impairs tissue function, most importantly in the central nervous system.

22.3.7. Animals Cannot Convert Fatty Acids into Glucose

It is important to note that *animals are unable to effect the net synthesis of glucose from fatty acids*. Specifically, acetyl CoA cannot be converted into pyruvate or oxaloacetate in animals. The two carbon atoms of the acetyl group of acetyl CoA enter the citric acid cycle, but two carbon atoms leave the cycle in the decarboxylations catalyzed by isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. Consequently, oxaloacetate is regenerated, but it is not formed de novo when the acetyl unit of acetyl CoA is oxidized by the citric acid cycle. In contrast, plants have two additional enzymes enabling them to convert the carbon atoms of acetyl CoA into oxaloacetate (Section 17.4.).

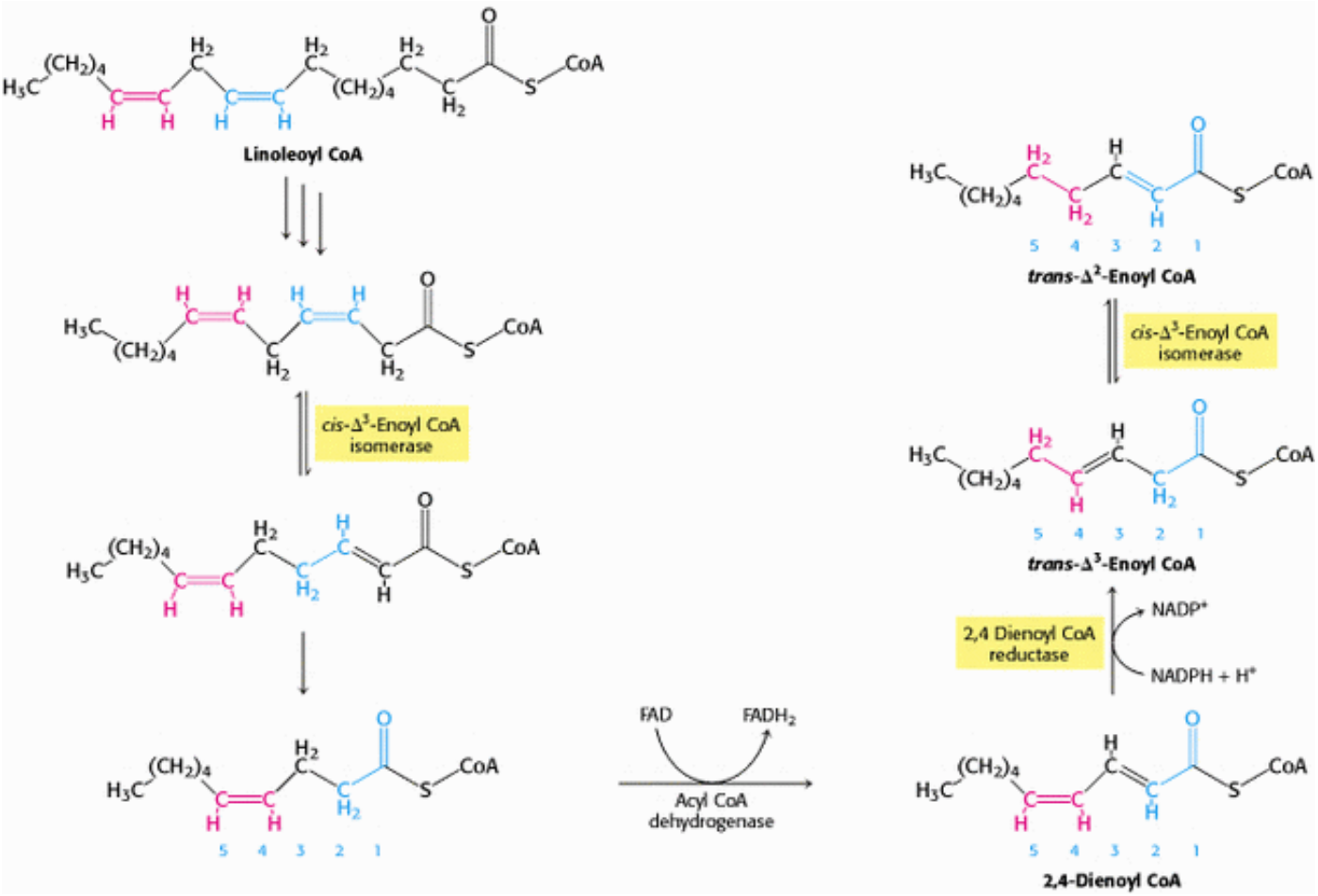


Figure 22.10. Oxidation of Linoleoyl CoA. The complete oxidation of the diunsaturated fatty acid linoleate is facilitated by the activity of enoyl CoA isomerase and 2,4-dienoyl CoA reductase.

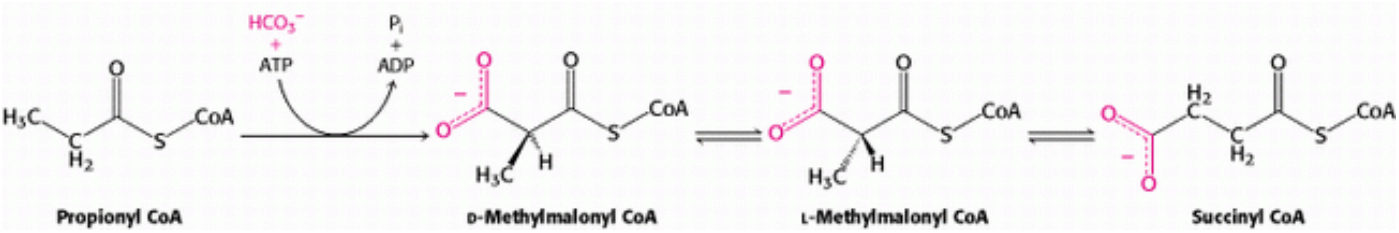


Figure 22.11. Conversion of Propionyl CoA Into Succinyl CoA. Propionyl CoA, generated from fatty acids with an odd number of carbons as well as some amino acids, is converted into the citric acid cycle intermediate succinyl CoA.

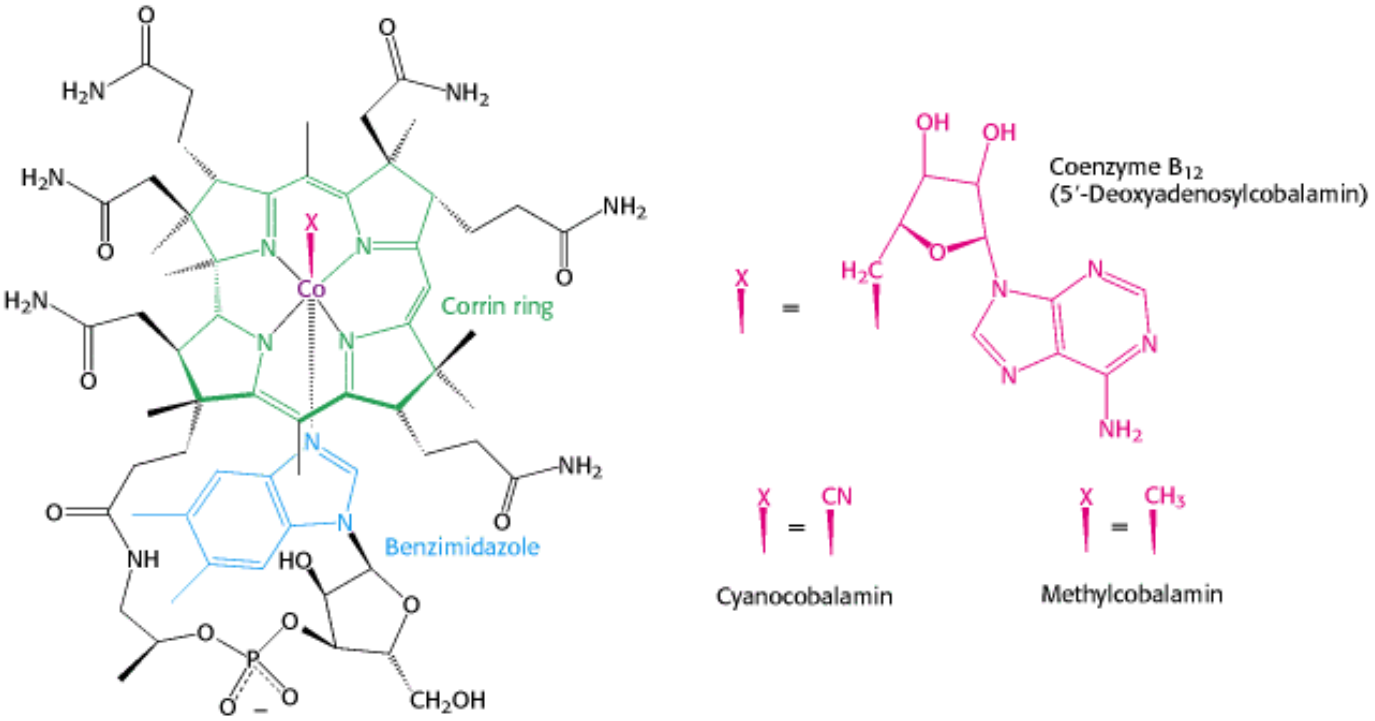


Figure 22.12. Structure of Coenzyme B_{12} (5′-deoxyadenosylcobalamin). Substitution of cyano and methyl groups create cyanocobalamin and methylcobalamin, respectively.

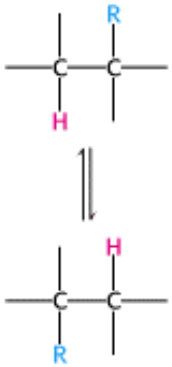


Figure 22.13. Rearrangement Reaction Catalyzed by Cobalamin Enzymes. The R group can be an amino group, a hydroxyl group, or a substituted carbon.

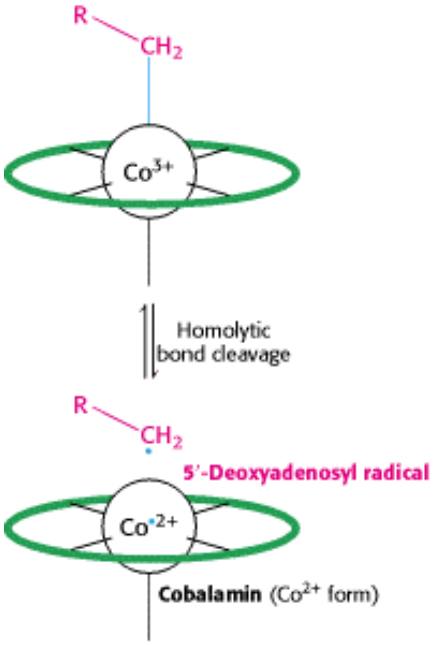


Figure 22.14. Formation of a 5′-Deoxyadenosyl Radical. The methylmalonyl CoA mutase reaction begins with the homolytic cleavage of the bond joining Co^{3+} to a carbon of the ribose of the adenosine moiety. The cleavage generates a 5′-deoxyadenosyl radical and leads to the reduction of Co^{3+} to Co^{2+}.

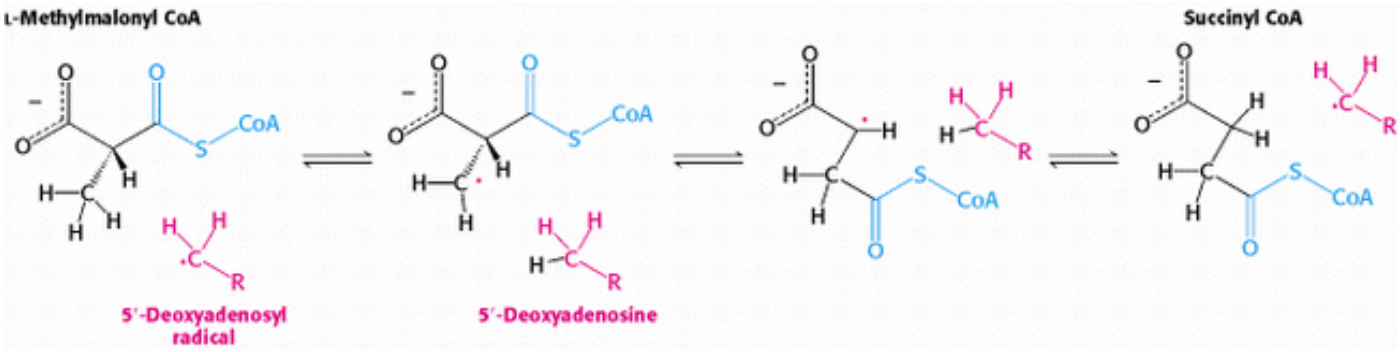


Figure 22.15. Formation of Succinyl CoA by a Rearrangement Reaction. A free radical abstracts a hydrogen atom in the rearrangement of methylmalonyl CoA to succinyl CoA.

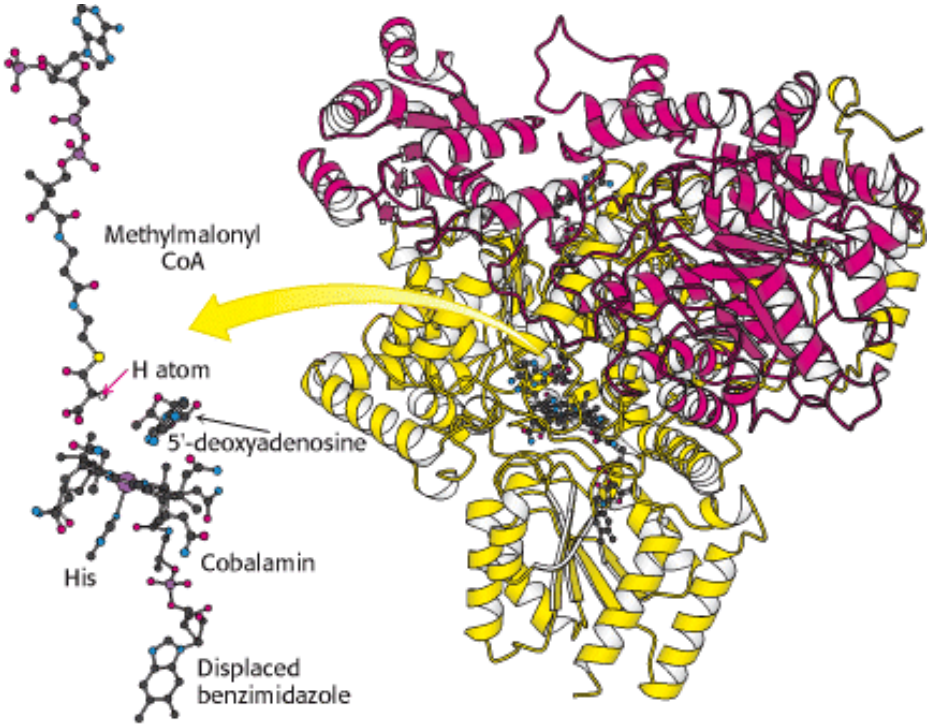


Figure 22.16. Active Site of Methylmalonyl CoA Mutase. The arrangement of substrate and coenzyme in the active site facilitates the cleavage of the cobalt-carbon bond and the subsequent abstraction of a hydrogen atom from the substrate.

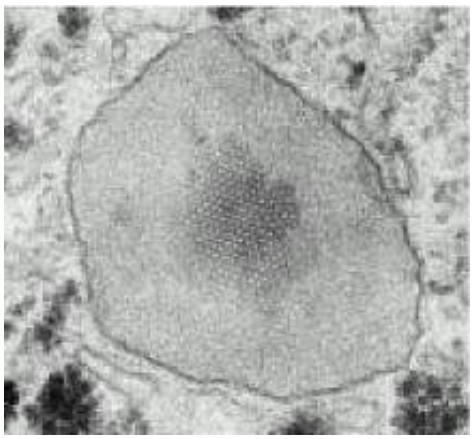


Figure 22.17. Electron Micrograph of a Peroxisome in a Liver Cell. A crystal of urate oxidase is present inside the organelle, which is bounded by a single bilayer membrane. The dark granular structures outside the peroxisome are glycogen particles. [Courtesy of Dr. George Palade.]

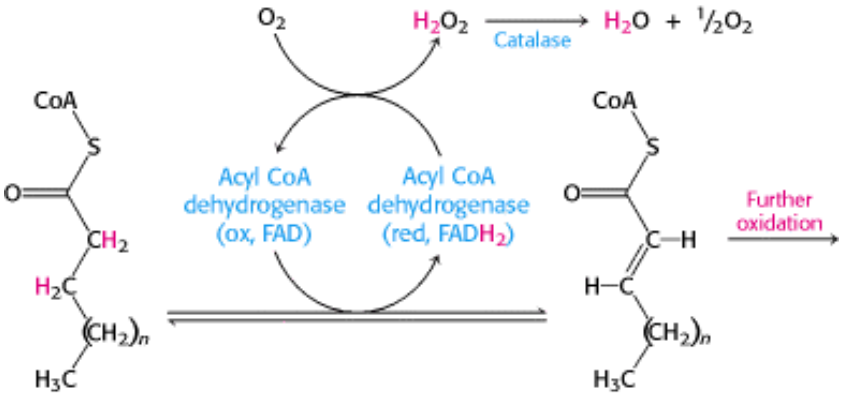


Figure 22.18. Initiation of Peroxisomal Fatty Acid Degradation. The first dehydration in the degradation of fatty acids in peroxisomes requires a flavoprotein dehydrogenase that transfers electrons to O_2 to yield H_2O_2.

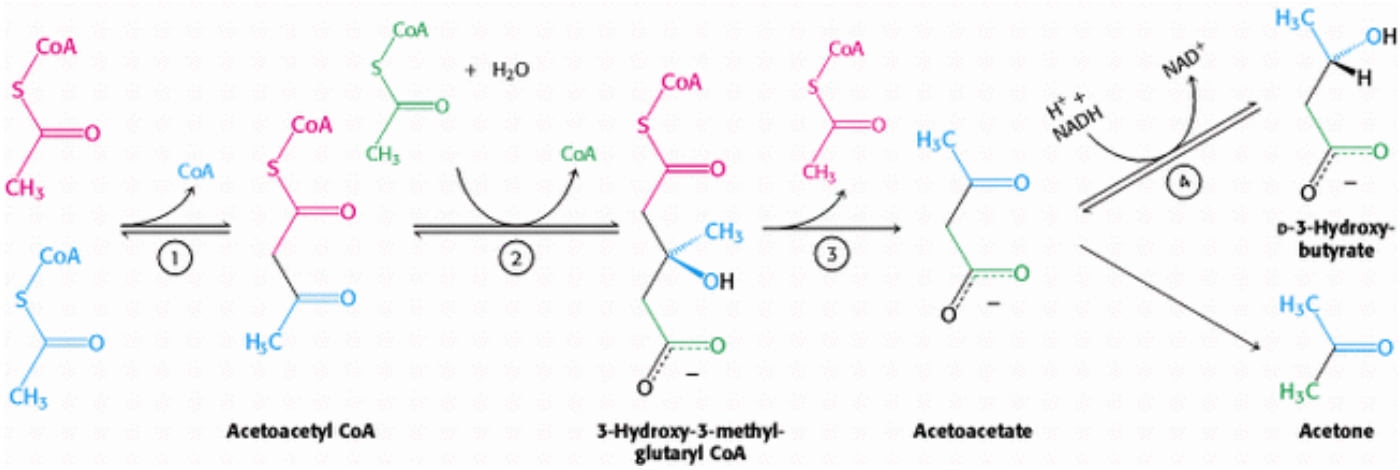


Figure 22.19. Formation of Ketone Bodies. The Ketone bodies-acetoacetate, D-3-hydroxybutyrate, and acetone from acetyl CoA—are formed primarily in the liver. Enzymes catalyzing these reactions are (1) 3-ketothiolase, (2) hydroxymethylglutaryl CoA synthase, (3) hydroxymethylglutaryl CoA cleavage enzyme, and (4) D-3-hydroxybutyrate dehydrogenase. Acetoacetate spontaneously decarboxylates to form acetone.

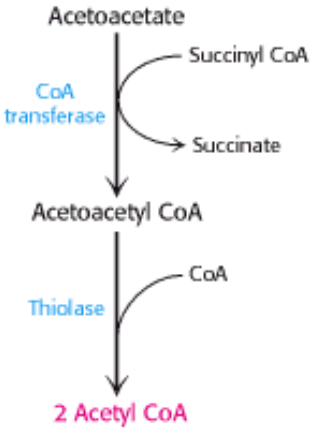


Figure 22.20. Utilization of Acetoacetate as a Fuel. Acetoacetate can be converted into two molecules of acetyl CoA, which then enter the citric acid cycle.

22.4. Fatty Acids Are Synthesized and Degraded by Different Pathways

Conceptual Insights, Overview of Carbohydrate and Fatty Acid Metabolism, will help you understand how fatty acid metabolism fits in with other energy storage and utilization pathways (glycolysis, citric acid cyclem, pentose phosphate pathwaym, glycogen metabolism), with a focus on carbon and energy flux.

Fatty acid synthesis is not simply a reversal of the degradative pathway. Rather, it consists of a new set of reactions, again exemplifying the principle that *synthetic and degradative pathways are almost always distinct*. Some important differences between the pathways are:

1. Synthesis takes place in the *cytosol*, in contrast with degradation, which takes place primarily in the mitochondrial matrix.

2. Intermediates in fatty acid synthesis are covalently linked to the sulfhydryl groups of an *acyl carrier protein* (ACP), whereas intermediates in fatty acid breakdown are covalently attached to the sulfhydryl group of coenzyme A.

3. The enzymes of fatty acid synthesis in higher organisms are joined in a *single polypeptide chain* called *fatty acid synthase*. In contrast, the degradative enzymes do not seem to be associated.

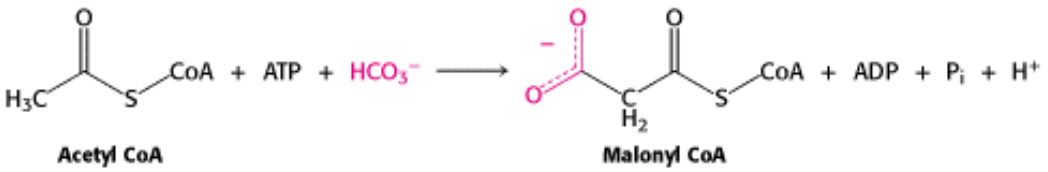
4. The growing fatty acid chain is elongated by the *sequential addition of two-carbon units* derived from acetyl CoA. The activated donor of twocarbon units in the elongation step is *malonyl ACP*. The elongation reaction is driven by the release of CO_2.

5. The reductant in fatty acid synthesis is *NADPH*, whereas the oxidants in fatty acid degradation are NAD^+ and *FAD*.

6. Elongation by the fatty acid synthase complex stops on formation of *palmitate* (C_{16}). Further elongation and the insertion of double bonds are carried out by other enzyme systems.

22.4.1. The Formation of Malonyl Coenzyme A Is the Committed Step in Fatty Acid Synthesis

Fatty acid synthesis starts with the carboxylation of acetyl CoA to *malonyl CoA*. This irreversible reaction is the committed step in fatty acid synthesis.



The synthesis of malonyl CoA is catalyzed by *acetyl CoA carboxylase*, which contains a biotin prosthetic group. The carboxyl group of biotin is covalently attached to the ε amino group of a lysine residue, as in pyruvate carboxylase (Section 16.3.2) and propionyl CoA carboxylase (Section 22.3.3). As with these other enzymes, a carboxybiotin intermediate is formed at the expense of the hydrolysis a molecule of ATP. The activated CO_2 group in this intermediate is then transferred to acetyl CoA to form malonyl CoA.

$$\text{Biotin-enzyme} + \text{ATP} + HCO_3^- \rightleftharpoons CO_2\text{-biotin-enzyme} + \text{ADP} + P_i$$

$$CO_2\text{-biotin-enzyme} + \text{acetyl CoA} \longrightarrow \text{malonyl CoA} + \text{biotin-enzyme}$$

This enzyme is also the essential regulatory enzyme for fatty acid metabolism (Section 22.5).

22.4.2. Intermediates in Fatty Acid Synthesis Are Attached to an Acyl Carrier Protein

The intermediates in fatty acid synthesis are linked to an acyl carrier protein. Specifically, they are linked to the sulfhydryl terminus of a phosphopantetheine group, which is, in turn, attached to a serine residue of the acyl carrier protein (Figure 22.21). Recall that, in the degradation of fatty acids, a phosphopantetheine group is present as part of CoA instead (Section 22.2.2). ACP, a single polypeptide chain of 77 residues, can be regarded as a giant prosthetic group, a "macro CoA."

22.4.3. The Elongation Cycle in Fatty Acid Synthesis

The enzyme system that catalyzes the synthesis of saturated long-chain fatty acids from acetyl CoA, malonyl CoA, and NADPH is called the *fatty acid synthase*. The constituent enzymes of bacterial fatty acid synthases are dissociated when the cells are broken apart. The availability of these isolated enzymes has facilitated the elucidation of the steps in fatty acid synthesis (Table 22.2). In fact, the reactions leading to fatty acid synthesis in higher organisms are very much like those of bacteria.

The elongation phase of fatty acid synthesis starts with the formation of acetyl ACP and malonyl ACP. *Acetyl transacylase* and *malonyl transacylase* catalyze these reactions.

$$\text{Acetyl CoA} + \text{ACP} \rightleftharpoons \text{acetyl ACP} + \text{CoA}$$

$$\text{Malonyl CoA} + \text{ACP} \rightleftharpoons \text{malonyl ACP} + \text{CoA}$$

Malonyl transacylase is highly specific, whereas acetyl transacylase can transfer acyl groups other than the acetyl unit, though at a much slower rate. Fatty acids with an odd number of carbon atoms are synthesized starting with propionyl ACP, which is formed from propionyl CoA by acetyl transacylase.

Acetyl ACP and malonyl ACP react to form acetoacetyl ACP (Figure 22.22). The *acyl-malonyl ACP condensing enzyme* catalyzes this condensation reaction.

$$\text{Acetyl ACP} + \text{malonyl ACP} \longrightarrow \text{acetoacetyl ACP} + \text{ACP} + CO_2$$

In the condensation reaction, a four-carbon unit is formed from a twocarbon unit and a three-carbon unit, and CO_2 is released. Why is the four-carbon unit not formed from 2 two-carbon units? In other words, why are the reactants acetyl ACP and malonyl ACP rather than two molecules of acetyl ACP? The answer is that the equilibrium for the synthesis of acetoacetyl ACP from two molecules of acetyl ACP is highly unfavorable. In contrast, *the equilibrium is favorable if malonyl ACP is a reactant because its decarboxylation contributes a substantial decrease in free energy*. In effect, ATP drives the condensation reaction, though ATP does not directly participate in the condensation reaction. Rather, ATP is used to carboxylate acetyl CoA to malonyl CoA. The free energy thus stored in malonyl CoA is released in the decarboxylation accompanying the formation of acetoacetyl ACP. Although HCO_3^- is required for fatty acid synthesis, its carbon atom does not appear in the product. Rather, *all the carbon atoms of fatty acids containing an even number of carbon atoms are derived from acetyl CoA*.

The next three steps in fatty acid synthesis reduce the keto group at C-3 to a methylene group (see Figure 22.22). First, acetoacetyl ACP is reduced to D-3-hydroxybutyryl ACP. This reaction differs from the corresponding one in fatty acid degradation in two respects: (1) the D rather than the L isomer is formed; and (2) NADPH is the reducing agent, whereas NAD^+ is the oxidizing agent in β oxidation. This difference exemplifies the general principle that *NADPH is consumed in biosynthetic reactions, whereas NADH is generated in energy-yielding reactions*. Then D-3-hydroxybutyryl ACP is *dehydrated* to form crotonyl ACP, which is a *trans*-Δ^2-enoyl ACP. The final step in the cycle *reduces* crotonyl ACP to butyryl ACP. NADPH is again the reductant, whereas FAD is the oxidant in the corresponding reaction in β-oxidation. The enzyme that catalyzes this step, *enoyl ACP reductase*, is inhibited by triclosan, a broad-spectrum antibacterial agent. Triclosan is used in a variety of products such as toothpaste, soaps, and skin creams. These last three reactions—a reduction, a dehydration, and a second reduction—convert acetoacetyl ACP into butyryl ACP, which completes the first elongation cycle.

In the second round of fatty acid synthesis, butyryl ACP condenses with malonyl ACP to form a C_6-β-ketoacyl ACP. This reaction is like the one in the first round, in which acetyl ACP condenses with malonyl ACP to form a C_4-β-ketoacyl ACP. Reduction, dehydration, and a second reduction convert the C_6-β-ketoacyl ACP into a C_6-acyl ACP, which is ready for a third round of elongation. The elongation cycles continue until C_{16}-acyl ACP is formed. This intermediate is a good substrate for a thioesterase that hydrolyzes C_{16}-acyl ACP to yield palmitate and ACP. *The thioesterase acts as a ruler to determine fatty acid chain length*. The synthesis of longer-chain fatty acids is discussed in Section 22.6.

22.4.4. Fatty Acids Are Synthesized by a Multifunctional Enzyme Complex in Eukaryotes

Although the basic biochemical reactions in fatty acid synthesis are very similar in *E. coli* and eukaryotes, the structure of the synthase varies considerably. The fatty acid synthases of eukaryotes, in contrast with those of *E. coli*, have the component enzymes linked in a large polypeptide chain.

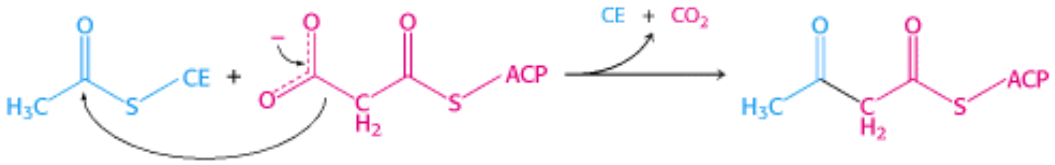
Mammalian fatty acid synthase is a dimer of identical 260-kd subunits. Each chain is folded into three domains joined by flexible regions (Figure 22.23). *Domain 1, the substrate entry and condensation unit*, contains acetyl transferase, malonyl transferase, and β-ketoacyl synthase (condensing enzyme). *Domain 2, the reduction unit*, contains the acyl carrier protein, β-ketoacyl reductase, dehydratase, and enoyl reductase. *Domain 3, the palmitate release unit*, contains the thioesterase. Thus, *seven different catalytic sites are present on a single polypeptide chain*. It is noteworthy that many eukaryotic multienzyme complexes are multifunctional proteins in which different enzymes are linked covalently. An advantage of this arrangement is that the synthetic activity of different enzymes is coordinated. In addition, a multienzyme complex consisting of covalently joined enzymes is more stable than one formed by noncovalent attractions. Furthermore, intermediates can be efficiently handed from one active site to another without leaving the assembly. It seems likely that multifunctional enzymes such as fatty acid synthase arose in eukaryotic evolution by exon shuffling (Section 5.6.2), because each of the component enzymes is recognizably homologous to its bacterial counterpart.

22.4.5. The Flexible Phosphopantetheinyl Unit of ACP Carries Substrate from One Active Site to Another

We next examine the coordinated functioning of the mammalian fatty acid synthase. Fatty acid synthesis begins with the transfer of the acetyl group of acetyl CoA first to a serine residue in the active site of acetyl transferase and then to the sulfur atom of a cysteine residue in the active site of the condensing enzyme on one chain of the dimeric enzyme. Similarly, the malonyl group is transferred from malonyl CoA first to a serine residue in the active site of malonyl transferase and then to the sulfur atom of the phosphopantetheinyl group of the acyl carrier protein on the *other* chain in the dimer. Domain 1 of each chain of this dimer interacts with domains 2 and 3 of the other chain. Thus, each of the two functional units of the synthase consists of domains formed by different chains. Indeed, the arenas of catalytic action are

the interfaces between domains on opposite chains.

Elongation begins with the joining of the acetyl unit on the condensing enzyme (CE) to a two-carbon part of the malonyl unit on ACP (Figure 22.24).



CO_2 is released and an acetoacetyl-*S*-phosphopantetheinyl unit is formed on ACP. The active-site sulfhydryl group on the condensing enzyme is restored. The acetoacetyl group is then delivered to three active sites in domain 2 of the opposite chain to reduce it to a butyryl unit. This saturated C_4 unit then migrates from the phosphopantetheinyl sulfur atom on ACP to the cysteine sulfur atom on the condensing enzyme. The synthase is now ready for another round of elongation. The butyryl unit on the condensing enzyme becomes linked to a two-carbon part of the malonyl unit on ACP to form a six-carbon unit on ACP, which undergoes reduction. Five more rounds of condensation and reduction produce a palmitoyl (C_{16}) chain on the condensing enzyme, which is hydrolyzed to palmitate by the thioesterase on domain 3 of the opposite chain. The migration of the growing fatty acyl chain back and forth between ACP and the condensing enzyme in each round of elongation is analogous to the translocations of growing peptide chains that take place in protein synthesis (Section 29.3.7).

The flexibility and 20-Å maximal length of the phosphopantetheinyl moiety are critical for the function of this multienzyme complex. The enzyme subunits need not undergo large structural rearrangements to interact with the substrate. Instead, the substrate is on a long, flexible arm that can reach each of the numerous active sites. Recall that biotin and lipoamide also are on long, flexible arms in their multienzyme complexes. The organization of the fatty acid synthases of higher organisms enhances the efficiency of the overall process because intermediates are directly transferred from one active site to the next.

22.4.6. The Stoichiometry of Fatty Acid Synthesis

The stoichiometry of the synthesis of palmitate is

$$\text{Acetyl CoA} + 7\ \text{malonyl CoA} + 14\ \text{NADPH} + 20\ \text{H}^+ \longrightarrow \text{palmitate} + 7\ \text{CO}_2 + 14\ \text{NADP}^+ + 8\ \text{CoA} + 6\ \text{H}_2\text{O}$$

The equation for the synthesis of the malonyl CoA used in the preceding reaction is

$$7\ \text{Acetyl CoA} + 7\ \text{CO}_2 + 7\ \text{ATP} \longrightarrow 7\ \text{malonyl CoA} + 7\ \text{ADP} + 7\ \text{P}_i + 14\ \text{H}^+$$

Hence, the overall stoichiometry for the synthesis of palmitate is

$$8\ \text{Acetyl CoA} + 7\ \text{ATP} + 14\ \text{NADPH} + 6\ \text{H}^+ \longrightarrow \text{palmitate} + 14\ \text{NADP}^+ + 8\ \text{CoA} + 6\ \text{H}_2\text{O} + 7\ \text{ADP} + 7\ \text{P}_i$$

22.4.7. Citrate Carries Acetyl Groups from Mitochondria to the Cytosol for Fatty Acid Synthesis

The synthesis of palmitate requires the input of 8 molecules of acetyl CoA, 14 molecules of NADPH, and 7 molecules of ATP. Fatty acids are synthesized in the cytosol, whereas acetyl CoA is formed from pyruvate in mitochondria. Hence, acetyl CoA must be transferred from mitochondria to the cytosol. Mitochondria, however, are not readily permeable to acetyl CoA. Recall that carnitine carries only long-chain fatty acids. *The barrier to acetyl CoA is bypassed by citrate, which carries acetyl groups across the inner mitochondrial membrane*. Citrate is formed in the mitochondrial matrix by the condensation of acetyl CoA with oxaloacetate (Figure 22.25). When present at high levels, citrate is transported to the cytosol, where it is cleaved by *ATP-citrate lyase*.

$$\text{Citrate} + \text{ATP} + \text{CoA} + \text{H}_2\text{O} \rightleftharpoons \text{acetyl CoA} + \text{ADP} + \text{P}_\text{i} + \text{oxaloacetate}$$

Lyases-

Enzymes catalyzing the cleavage of C-C, C-O, or C-N bonds by elimination. A double bond is formed in these reactions.

Thus, acetyl CoA and oxaloacetate are transferred from mitochondria to the cytosol at the expense of the hydrolysis of a molecule of ATP.

22.4.8. Sources of NADPH for Fatty Acid Synthesis

Oxaloacetate formed in the transfer of acetyl groups to the cytosol must now be returned to the mitochondria. The inner mitochondrial membrane is impermeable to oxaloacetate. Hence, a series of bypass reactions are needed. Most important, these reactions generate much of the NADPH needed for fatty acid synthesis. First, oxaloacetate is reduced to malate by NADH. This reaction is catalyzed by a *malate dehydrogenase* in the cytosol.

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{malate} + \text{NAD}^+$$

Second, malate is oxidatively decarboxylated by an *$NADP^+$-linked malate enzyme* (also called *malic enzyme*).

$$\text{Malate} + \text{NADP}^+ \rightleftharpoons \text{pyruvate} + \text{CO}_2 + \text{NADPH}$$

The pyruvate formed in this reaction readily enters mitochondria, where it is carboxylated to oxaloacetate by pyruvate carboxylase.

$$\text{Pyruvate} + \text{CO}_2 + \text{ATP} + \text{H}_2\text{O} \rightleftharpoons \text{oxaloacetate} + \text{ADP} + \text{P}_\text{i} + 2\,\text{H}^+$$

The sum of these three reactions is

$$NADP^+ + NADH + ATP + H_2O \rightleftharpoons NADPH + NAD^+ + ADP + P_i + H^+$$

Thus, *one molecule of NADPH is generated for each molecule of acetyl CoA that is transferred from mitochondria to the cytosol*. Hence, eight molecules of NADPH are formed when eight molecules of acetyl CoA are transferred to the cytosol for the synthesis of palmitate. *The additional six molecules of NADPH required for this process come from the pentose phosphate pathway* (Section 20.3.1).

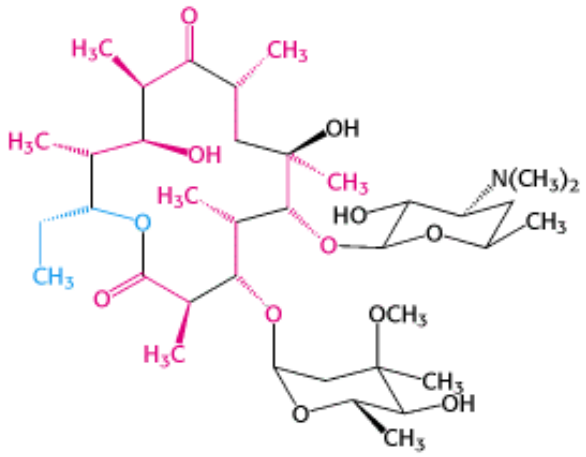
The accumulation of the precursors for fatty acid synthesis is a wonderful example of the coordinated use of multiple processes to fulfill a biochemical need. The citric acid cycle, subcellular compartmentalization, and the pentose phosphate pathway provide the carbon atoms and reducing power, whereas glycolysis and oxidative phosphorylation provide the ATP to meet the needs for fatty acid synthesis.

22.4.9. Fatty Acid Synthase Inhibitors May Be Useful Drugs

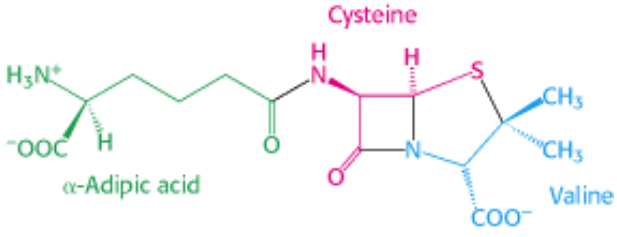
Fatty acid synthase is overexpressed in some breast cancers. Researchers intrigued by this observation have tested inhibitors of fatty acid synthase on mice to see how the inhibitors affect tumor growth. A startling observation was made: *mice treated with inhibitors of the condensing enzyme showed remarkable weight loss* due to inhibition of feeding. The results of additional studies revealed that this inhibition is due, at least in part, to the accumulation of malonyl CoA. Thus, fatty acid synthase inhibitors are exciting candidates both as antitumor and as antiobesity drugs.

22.4.10. Variations on a Theme: Polyketide and Nonribosomal Peptide Synthetases Resemble Fatty Acid Synthase

The mammalian multifunctional fatty acid synthase is a member of a large family of complex enzymes termed *megasynthases* that participate in step-by-step synthetic pathways. Two important classes of compounds that are synthesized by such enzymes are the *polyketides* and the *nonribosomal peptides*. The antibiotic erythromycin is an example of a polyketide, whereas penicillin (Section 8.5.5) is a nonribosomal peptide.



Erythromycin



Penicillin

The core of erythromycin (deoxyerythronolide B, or Deb) is synthesized by the following reaction:

$$\text{Propionyl CoA} + 6\,\text{methylmalonyl CoA} + 6\,NADPH + 6\,H^+ \longrightarrow \text{Deb} + 7\,\text{CoA} + 6\,NADP^+ + 2\,H_2O$$

This reaction is accomplished by three megasynthases consisting of 3491, 3567, and 3172 amino acids. The synthesis of deoxyerythronolide B begins with propionyl CoA linked to a phosphopantetheine chain connected to an acyl carrier protein domain. Similarly, the precursor of penicillin [Δ-(l-aminoadipyl)-l-cysteinyl-d-valine, or ACV] is generated by the following reaction:

$$\text{L-Aminoadipic acid} + \text{L-Cys} + \text{L-Val} + 3\,\text{ATP} + H_2O \longrightarrow \text{ACV} + 3\,\text{ADP} + 3\,P_i$$

which is catalyzed by a megasynthase consisting of 3791 amino acids. Each amino acid is activated by a specific adenylation domain within the enzyme—a domain that is homologous to acyl CoA synthase. Additional domains are responsible for peptide-bond formation and for the epimerization of the valine residue. Again, during synthesis, the components are linked to phosphopantetheine chains. Members of this remarkably modular enzyme family generate many of the natural products that have proved to be useful as drugs.

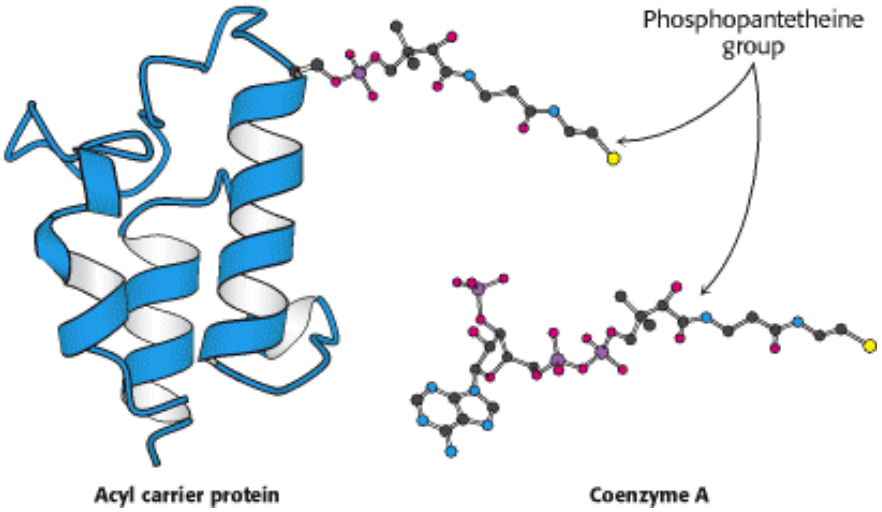


Figure 22.21. Phosphopantetheine. Both acyl carrier protein and CoA include phosphopantetheine as their reactive units.

Table 22.2. Principal reactions in fatty acid synthesis in bacteria

Step	Reaction	Enzyme
1	Acetyl CoA + HCO_3^- + ATP $\rightarrow$ malonyl CoA + ADP + P_i + H^+	Acetyl CoA carboxylase
2	Acetyl CoA + ACP $\rightleftharpoons$ acetyl ACP + CoA	Acetyl transacylase
3	Malonyl CoA + ACP $\rightleftharpoons$ malonyl ACP + CoA	Malonyl transacylase
4	Acetyl ACP + malonyl ACP $\rightarrow$ acetoacetyl ACP + ACP + CO_2	Acyl-malonyl ACP condensing enzyme
5	Acetoacetyl ACP + NADPH + H^+ $\rightleftharpoons$ d-3-hydroxybutyryl ACP + $NADP^+$	β-Ketoacyl ACP reductase
6	d-3-Hydroxybutyryl ACP $\rightleftharpoons$ crotonyl ACP + H_2O	3-Hydroxyacyl ACP dehydratase
7	Crotonyl ACP + NADPH + H^+ $\rightarrow$ butyryl ACP + $NADP^+$	Enoyl ACP reductase

Figure 22.22. Fatty Acid Synthesis. Fatty acids are synthesized by the repetition of the following reaction sequence: condensation, reduction, dehydration, and reduction. The intermediates shown here are produced in the first round of synthesis.

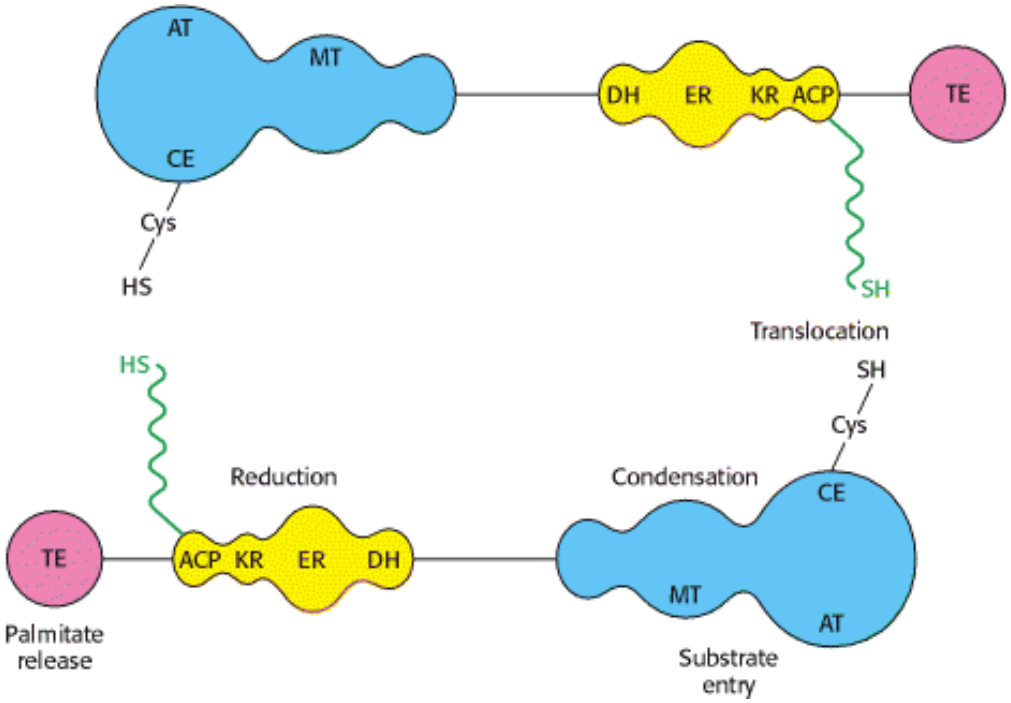


Figure 22.23. Schematic Representation of Animal Fatty Acid Synthase. Each of the identical chains in the dimer contains three domains. Domain 1 (blue) contains acetyl transferase (AT), malonyl transferase (MT), and condensing enzyme (CE). Domain 2 (yellow) contains acyl carrier protein (ACP), β-ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER). Domain 3 (red) contains thioesterase (TE). The flexible phosphopantetheinyl group (green) carries the fatty acyl chain from one catalytic site on a chain to another, as well as between chains in the dimer. [After Y. Tsukamoto, H. Wong, J. S. Mattick, and S. J. Wakil. *J. Biol. Chem.* 258(1983):15312.]

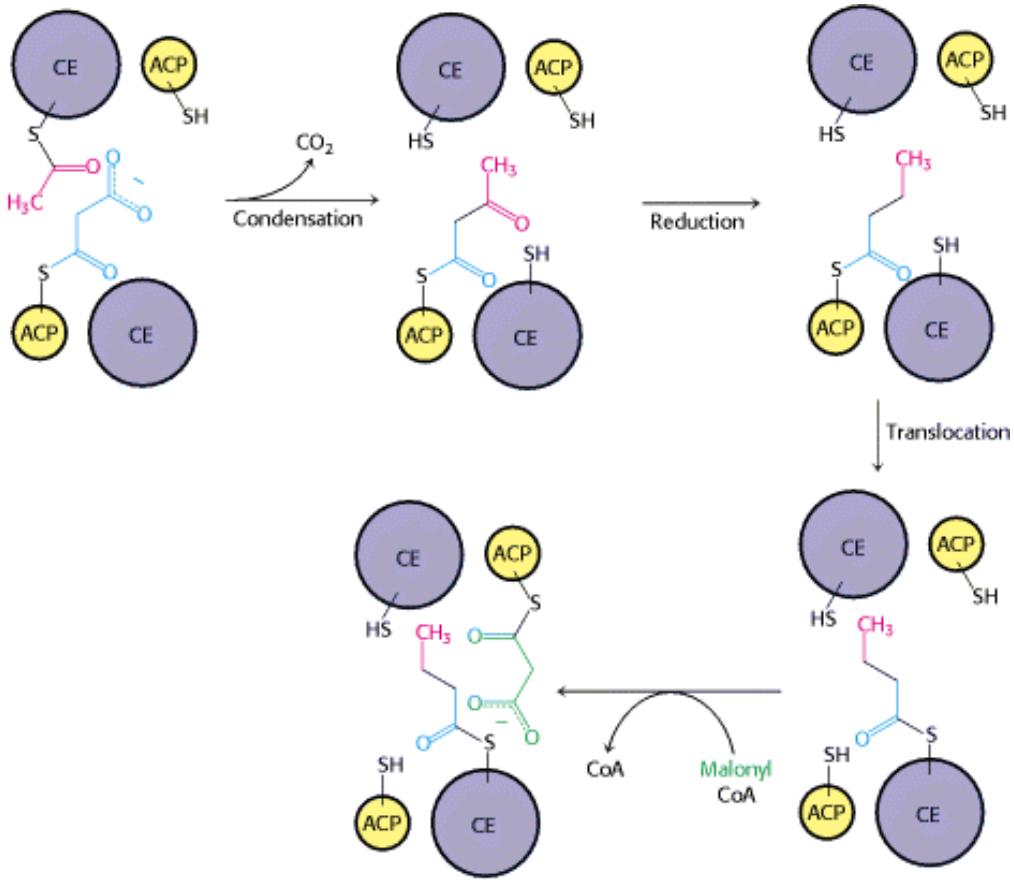


Figure 22.24. Reactions of Fatty Acid Synthase. Translocations of the elongating fatty acyl chain between the cysteine

sulfhydryl group of the condensing enzyme (CE, blue) and the phosphopantetheine sulfhydryl group of the acyl carrier protein (ACP, yellow) lead to the growth of the fatty acid chain. The reactions are repeated until the palmitoyl product is synthesized.

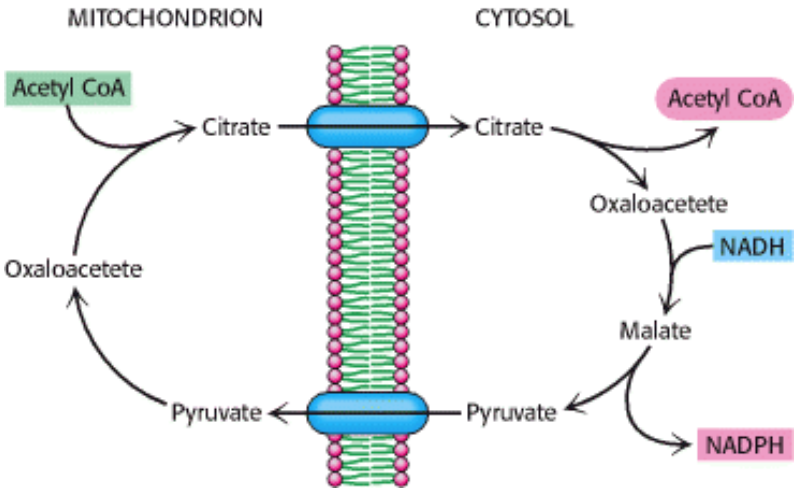


Figure 22.25. Transfer of Acetyl CoA to the Cytosol. Acetyl CoA is transferred from mitochondria to the cytosol, and the reducing potential NADH is concomitantly converted into that of NADPH by this series of reactions.

22.5. Acetyl Coenzyme A Carboxylase Plays a Key Role in Controlling Fatty Acid Metabolism

Fatty acid metabolism is stringently controlled so that synthesis and degradation are highly responsive to physiological needs. Fatty acid synthesis is maximal when carbohydrate and energy are plentiful and when fatty acids are scarce. *Acetyl CoA carboxylase plays an essential role in regulating fatty acid synthesis and degradation*. Recall that this enzyme catalyzes the committed step in fatty acid synthesis: the production of malonyl CoA (the activated two-carbon donor). The carboxylase is controlled by three global signals—glucagon, epinephrine, and insulin—that correspond to the overall energy status of the organism. *Insulin stimulates fatty acid synthesis by activating the carboxylase, whereas glucagon and epinephrine have the reverse effect*. The levels of citrate, palmitoyl CoA, and AMP within a cell also exert control. *Citrate*, a signal that building blocks and energy are abundant, activates the carboxylase. Palmitoyl CoA and AMP, in contrast, lead to the inhibition of the carboxylase. Thus, this important enzyme is subject to both global and local regulation. We will examine each of these levels of regulation in turn.

Global Regulation.

Global regulation is carried out by means of reversible phosphorylation. *Acetyl CoA carboxylase is switched off by phosphorylation* and activated by dephosphorylation (Figure 22.26). Modification of a single serine residue by an *AMP-dependent protein kinase (AMPK)* converts the carboxylase into an inactive form. The phosphoryl group on the inhibited carboxylase is removed by *protein phosphatase 2A*. The proportion of carboxylase in the active dephosphorylated form depends on the relative rates of these opposing enzymes.

How is the formation of the inactive, phosphorylated form of the enzyme regulated? AMPK, the enzyme that phosphorylates the carboxylase, is essentially a fuel gauge—it is activated by AMP and inhibited by ATP. Thus, the carboxylase is inactivated when the energy charge is low. This kinase is conserved among eukaryotes. Homologs found in yeast and plants also play roles in sensing the energy status of the cell. The inhibition of phosphatase 2A is necessary to maintain acetyl CoA carboxylase in the phosphorylated state. Epinephrine and glucagon activate protein kinase A, which in turn inhibits the phosphatase by phosphorylating it. Hence, *these catabolic hormones switch off fatty acid synthesis by keeping the carboxylase in the inactive phosphorylated state*.

How is the enzyme dephosphorylated and activated? *Insulin stimulates the carboxylase by causing its dephosphorylation*. It is not clear which of the phosphatases activates the carboxylase in response to insulin. The hormonal control of acetyl CoA carboxylase is reminiscent of that of glycogen synthase (Section 21.5.2).

Local Regulation.

Acetyl CoA carboxylase is also under local control. *This enzyme is allosterically stimulated by citrate*. Specifically, citrate partly reverses the inhibition produced by phosphorylation. It acts in an unusual manner on inactive acetyl CoA carboxylase, which exists as an octamer (Figure 22.27). *Citrate facilitates the polymerization of the inactive octamers into active filaments* (Figure 22.28). The level of citrate is high when both acetyl CoA and ATP are abundant. Recall that mammalian isocitrate dehydrogenase is inhibited by a high energy charge (Section 17.2.2). Hence, a high level of citrate signifies that two-carbon units and ATP are available for fatty acid synthesis. The stimulatory effect of citrate on the carboxylase is antagonized by *palmitoyl CoA*, which is abundant when there is an excess of fatty acids. Palmitoyl CoA causes the filaments to disassemble into the inactive octamers. Palmitoyl CoA also inhibits the translocase that transports citrate from mitochondria to the cytosol, as well as glucose 6-phosphate dehydrogenase, which generates NADPH in the pentose phosphate pathway.

Response to Diet.

Fatty acid synthesis and degradation are reciprocally regulated so that both are not simultaneously active. *In starvation, the level of free fatty acids rises because hormones such as epinephrine and glucagon stimulate adipose-cell lipase. Insulin, in contrast, inhibits lipolysis*. Acetyl CoA carboxylase also plays a role in the regulation of fatty acid degradation. Malonyl CoA, the product of the carboxylase reaction, is present at a high level when fuel molecules are abundant. *Malonyl CoA inhibits carnitine acyltransferase I, preventing access of fatty acyl CoAs to the mitochondrial matrix in times of plenty*. Malonyl CoA is an especially effective inhibitor of carnitine acyltransferase I in heart and muscle, tissues that have little fatty acid synthesis capacity of their own. In these tissues, acetyl CoA carboxylase may be a purely regulatory enzyme. Finally, two enzymes in the β-oxidation pathway are markedly inhibited when the energy charge is high. NADH inhibits 3-hydroxyacyl CoA dehydrogenase, and acetyl CoA inhibits thiolase.

Long-term control is mediated by changes in the rates of synthesis and degradation of the enzymes participating in fatty acid synthesis. Animals that have fasted and are then fed high-carbohydrate, low-fat diets show marked increases in their amounts of acetyl CoA carboxylase and fatty acid synthase within a few days. This type of regulation is known as *adaptive control*.

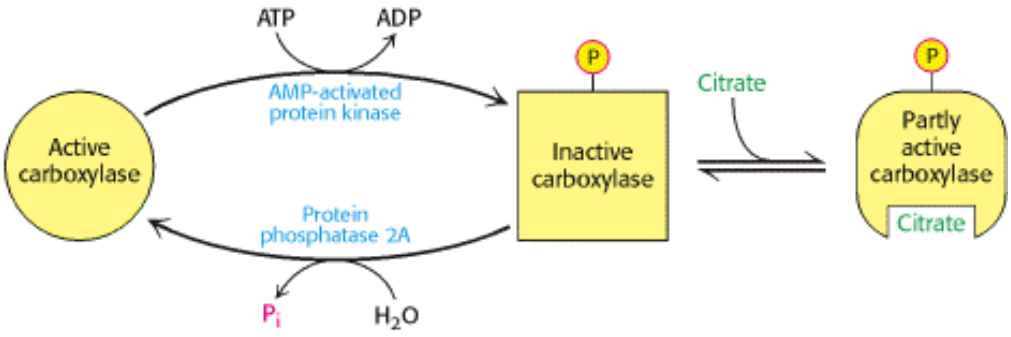


Figure 22.26. Control of Acetyl CoA Carboxylase. Acetyl CoA carboxylase is inhibited by phosphorylation and activated by the binding of citrate.

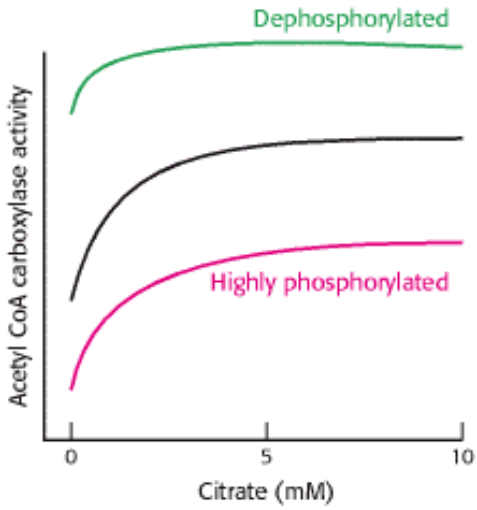


Figure 22.27. Dependence of the Catalytic Activity of Acetyl CoA Carboxylase on the Concentration of Citrate. The dephosphorylated form of the carboxylase is highly active even when citrate is absent. Citrate partly overcomes the inhibition produced by phosphorylation. [After G. M. Mabrouk, I. M. Helmy, K. G. Thampy, and S. J. Wakil. *J. Biol. Chem.* 265(1990):6330.]

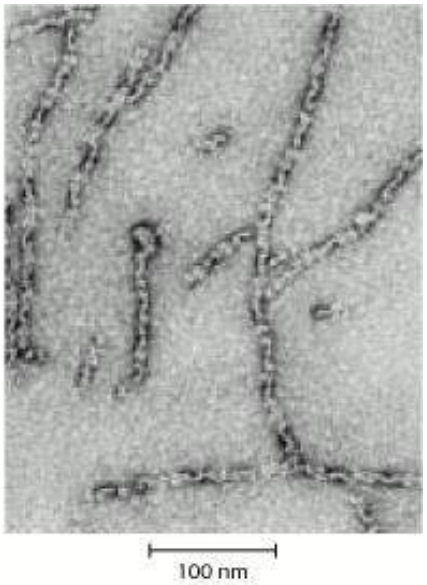


Figure 22.28. Filaments of Acetyl CoA Carboxylase. The electron micrograph shows the enzymatically active filamentous form of acetyl CoA carboxylase from chicken liver. The inactive form is an octamer of 265-kd subunits. [Courtesy of Dr. M. Daniel Lane.]

22.6. Elongation and Unsaturation of Fatty Acids Are Accomplished by Accessory Enzyme Systems

The major product of the fatty acid synthase is palmitate. In eukaryotes, longer fatty acids are formed by elongation reactions catalyzed by enzymes on the cytosolic face of the *endoplasmic reticulum membrane*. These reactions add two-carbon units sequentially to the carboxyl ends of both saturated and unsaturated fatty acyl CoA substrates. Malonyl CoA is the two-carbon donor in the elongation of fatty acyl CoAs. Again, condensation is driven by the decarboxylation of malonyl CoA.

22.6.1. Membrane-Bound Enzymes Generate Unsaturated Fatty Acids

Endoplasmic reticulum systems also introduce double bonds into long-chain acyl CoAs. For example, in the conversion of stearoyl CoA into oleoyl CoA, a cis-Δ^{9} double bond is inserted by an oxidase that employs *molecular oxygen* and *NADH* (or *NADPH*).

$$\text{Stearoyl CoA} + \text{NADH} + \text{H}^{+} + \text{O}_2 \longrightarrow \text{oleoyl CoA} + \text{NAD}^{+} + 2\,\text{H}_2\text{O}$$

This reaction is catalyzed by a complex of three membrane-bound enzymes: *NADH-cytochrome* b_5 *reductase, cytochrome* b_5, and a *desaturase* (Figure 22.29). First, electrons are transferred from NADH to the FAD moiety of NADH-cytochrome b_5 reductase.

The heme iron atom of cytochrome b_5 is then reduced to the Fe^{2+} state. The nonheme iron atom of the desaturase is subsequently converted into the Fe^{2+} state, which enables it to interact with O_2 and the saturated fatty acyl CoA substrate. A double bond is formed and two molecules of H_2O are released. Two electrons come from NADH and two from the single bond of the fatty acyl substrate.

A variety of unsaturated fatty acids can be formed from oleate by a combination of elongation and desaturation reactions. For example, oleate can be elongated to a 20:1 cis-Δ^{11} fatty acid. Alternatively, a second double bond can be inserted to yield an 18:2 cis-Δ^{6},Δ^{9} fatty acid. Similarly, palmitate (16:0) can be oxidized to palmitoleate (16:1 cis-Δ^{9}), which can then be elongated to *cis*-vaccenate (18:1 cis-Δ^{11}).

Unsaturated fatty acids in mammals are derived from either palmitoleate (16:1), oleate (18:1), linoleate (18:2), or linolenate (18:3). The number of carbon atoms from the ω end of a derived unsaturated fatty acid to the nearest double bond identifies its precursor.

Precursor	Formula
Linolenate (ω-3)	$CH_3–(CH_2)_1–CH=CH–R$
Linoleate (ω-6)	$CH_3–(CH_2)_4–CH=CH–R$
Palmitoleate (ω-7)	$CH_3–(CH_2)_5–CH=CH–R$
Oleate (ω-9)	$CH_3–(CH_2)_7–CH=CH–R$

Mammals lack the enzymes to introduce double bonds at carbon atoms beyond C-9 in the fatty acid chain. Hence, mammals cannot synthesize linoleate (18:2 cis-Δ^{9}, Δ^{12}) and linolenate (18:3 cis-Δ^{9}, Δ^{12}, Δ^{15}). *Linoleate and linolenate are the two essential fatty acids*. The term *essential* means that they must be supplied in the diet because they are required by an organism and cannot be endogenously synthesized. Linoleate and linolenate furnished by the diet are the starting points for the synthesis of a variety of other unsaturated fatty acids.

22.6.2. Eicosanoid Hormones Are Derived from Polyunsaturated Fatty Acids

Arachidonate, a 20:4 fatty acid derived from linoleate, is the major precursor of several classes of signal molecules: prostaglandins, prostacyclins, thromboxanes, and leukotrienes (Figure 22.30).

A prostaglandin is a 20-carbon fatty acid containing a 5-carbon ring (Figure 22.31). A series of prostaglandins is fashioned by reductases and isomerases. The major classes are designated PGA through PGI; a subscript denotes the number of carbon-carbon double bonds outside the ring. Prostaglandins with two double bonds, such as PGE_2, are derived from arachidonate; the other two double bonds of this precursor are lost in forming a five-membered ring. *Prostacyclin* and *thromboxanes* are related compounds that arise from a nascent prostaglandin. They are generated by *prostacylin synthase* and *thromboxane synthase* respectively. Alternatively, arachidonate can be converted into *leukotrienes* by the action of *lipoxygenase*. These compounds, first found in leukocytes, contain three conjugated double bonds—hence, the name. Prostaglandins, prostacyclin, thromboxanes, and leukotrienes are called *eicosanoids* (from the Greek *eikosi*, "twenty") because they contain 20 carbon atoms.

Prostaglandins and other eicosanoids are *local hormones* because they are short-lived. They alter the activities both of the cells in which they are synthesized and of adjoining cells by binding to 7TM receptors. The nature of these effects may vary from one type of cell to another, in contrast with the more uniform actions of global hormones such as insulin and glucagon. Prostaglandins stimulate inflammation, regulate blood flow to particular organs, control ion transport across membranes, modulate synaptic transmission, and induce sleep.


Recall that aspirin blocks access to the active site of the enzyme that converts arachidonate into prostaglandin H_2 (Section 12.5.2). Because arachidonate is the precursor of other prostaglandins, prostacyclin, and thromboxanes, blocking this step affects many signaling pathways. It accounts for the wide-ranging effects that aspirin and related compounds have on inflammation, fever, pain, and blood clotting.

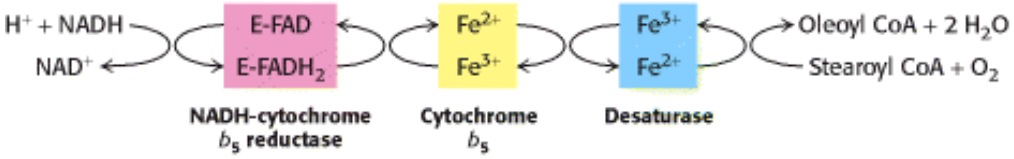


Figure 22.29. Electron-Transport Chain in the Desaturation of Fatty Acids.

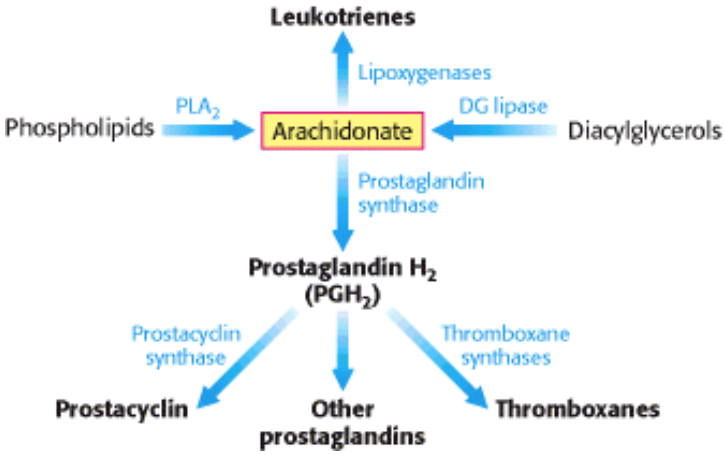


Figure 22.30. Arachidonate Is the Major Precursor of Eicosanoid Hormones. Prostaglandin synthase catalyzes the first step in a pathway leading to prostaglandins, prostacyclins, and thromboxanes. Lipoxygenase catalyzes the initial step in a pathway leading to leukotrienes.

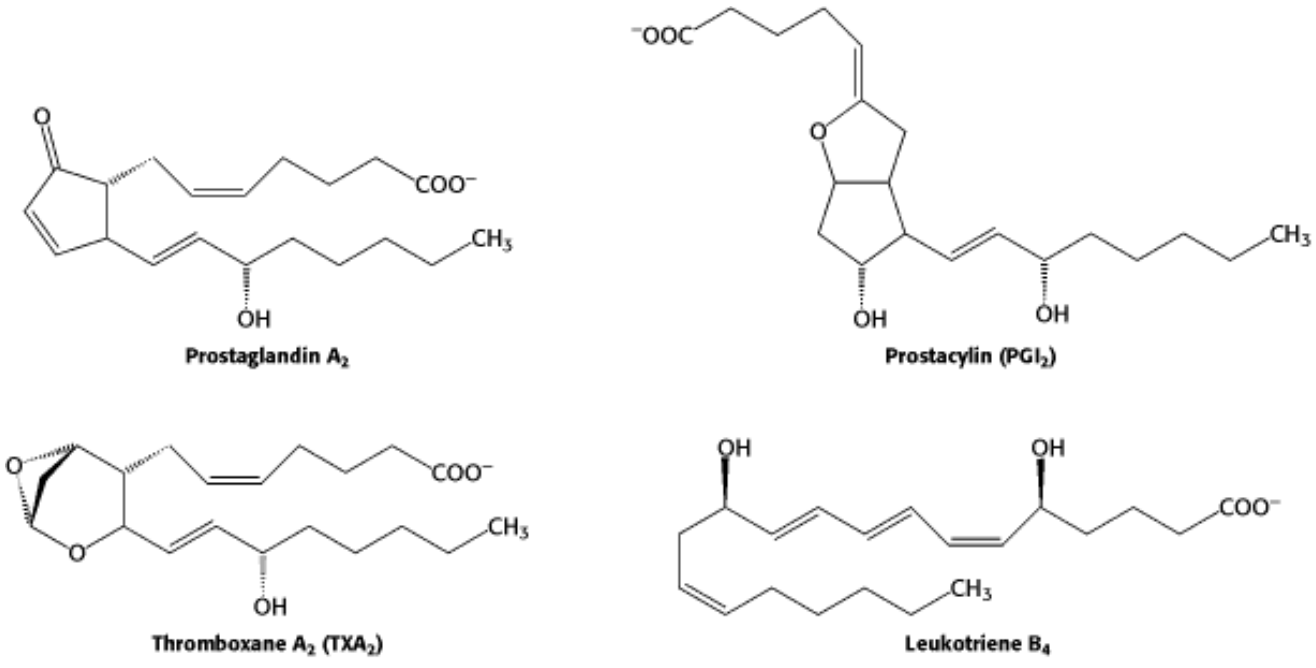


Figure 22.31. Structures of Several Eicosanoids.

Summary

Triacylglycerols Are Highly Concentrated Energy Stores

Fatty acids are physiologically important as (1) components of phospholipids and glycolipids, (2) hydrophilic modifiers of proteins, (3) fuel molecules, and (4) hormones and intracellular messengers. They are stored in adipose tissue as triacylglycerols (neutral fat).

The Utilization of Fatty Acids as Fuel Requires Three Stages of Processing

Triacylglycerols can be mobilized by the hydrolytic action of lipases that are under hormonal control. Fatty acids are activated to acyl CoAs, transported across the inner mitochondrial membrane by carnitine, and degraded in the mitochondrial matrix by a recurring sequence of four reactions: oxidation by FAD, hydration, oxidation by NAD^+, and thiolysis by CoA. The $FADH_2$ and NADH formed in the oxidation steps transfer their electrons to O_2 by means of the respiratory chain, whereas the acetyl CoA formed in the thiolysis step normally enters the citric acid cycle by condensing with oxaloacetate. Mammals are unable to convert fatty acids into glucose, because they lack a pathway for the net production of oxaloacetate, pyruvate, or other gluconeogenic intermediates from acetyl CoA.

Certain Fatty Acids Require Additional Steps for Degradation

Fatty acids that contain double bonds or odd numbers of carbon atoms require ancillary steps to be degraded. An isomerase and a reductase are required for the oxidation of unsaturated fatty acids, whereas propionyl CoA derived from chains with odd numbers of carbons requires a vitamin B_{12}-dependent enzyme to be converted into succinyl CoA.

Fatty Acids Are Synthesized and Degraded by Different Pathways

Fatty acids are synthesized in the cytosol by a different pathway from that of β oxidation. Synthesis starts with the carboxylation of acetyl CoA to malonyl CoA, the committed step. This ATP-driven reaction is catalyzed by acetyl CoA carboxylase, a biotin enzyme. The intermediates in fatty acid synthesis are linked to an acyl carrier protein. Acetyl ACP is formed from acetyl CoA, and malonyl ACP is formed from malonyl CoA. Acetyl ACP and malonyl ACP condense to form acetoacetyl ACP, a reaction driven by the release of CO_2 from the activated malonyl unit. A reduction, a dehydration, and a second reduction follow. NADPH is the reductant in these steps. The butyryl ACP formed in this way is ready for a second round of elongation, starting with the addition of a two-carbon unit from malonyl ACP. Seven rounds of elongation yield palmitoyl ACP, which is hydrolyzed to palmitate. In higher organisms, the enzymes carrying out fatty acid synthesis are covalently linked in a multifunctional enzyme complex. A reaction cycle based on the formation and cleavage of citrate carries acetyl groups from mitochondria to the cytosol. NADPH needed for synthesis is generated in the transfer of reducing equivalents from mitochondria by the malate-pyruvate shuttle and by the pentose phosphate pathway.

Acetyl Coenzyme A Carboxylase Plays a Key Role in Controlling Fatty Acid Metabolism

Fatty acid synthesis and degradation are reciprocally regulated so that both are not simultaneously active. Acetyl CoA carboxylase, the essential control site, is stimulated by insulin and inhibited by glucagon and epinephrine. These hormonal effects are mediated by changes in the amounts of the active dephosphorylated and inactive phosphorylated forms of the carboxylase. Citrate, which signals an abundance of building blocks and energy, allosterically stimulates the carboxylase. Glucagon and epinephrine stimulate triacylglycerol breakdown by activating the lipase. Insulin, in contrast, inhibits lipolysis. In times of plenty, fatty acyl CoAs do not enter the mitochondrial matrix because malonyl CoA inhibits

carnitine acyltransferase I.

Elongation and Unsaturation of Fatty Acids Are Accomplished by Accessory Enzyme Systems

Fatty acids are elongated and desaturated by enzyme systems in the endoplasmic reticulum membrane. Desaturation requires NADH and O_2 and is carried out by a complex consisting of a flavoprotein, a cytochrome, and a nonheme iron protein. Mammals lack the enzymes to introduce double bonds distal to C-9, and so they require linoleate and linolenate in their diets.

Arachidonate, an essential precursor of prostaglandins and other signal molecules, is derived from linoleate. This 20:4 polyunsaturated fatty acid is the precursor of several classes of signal molecules—prostaglandins, prostacyclins, thromboxanes, and leukotrienes—that act as messengers and local hormones because of their transience. They are called eicosanoids because they contain 20 carbon atoms. Prostaglandin synthase, the enzyme catalyzing the first step in the synthesis of all eicosanoids except the leukotrienes, consists of a cyclooxygenase and a hydroperoxidase. Aspirin (acetylsalicylate), an anti-inflammatory and antithrombotic drug, irreversibly blocks the synthesis of these eicosanoids.

Key Terms

triacylglycerol (neutral fat, triacylglyceride)

bile salt

chylomicron

acyl adenylate

carnitine

β-oxidation pathway

vitamin B_{12} (cobalamin)

peroxisome

ketone body

acyl carrier protein (ACP)

fatty acid synthase

malonyl CoA

acetyl CoA carboxylase

megasynthase

polyketide

nonribosomal peptide

AMP-dependent protein kinase (AMPK)

protein phosphatase 2A

arachidonate

prostaglandin

eicosanoid

Problems

1. *After lipolysis*. Write a balanced equation for the conversion of glycerol into pyruvate. Which enzymes are required in addition to those of the glycolytic pathway?

See answer

2. *From fatty acid to ketone body*. Write a balanced equation for the conversion of stearate into acetoacetate.

See answer

3. *Counterpoint*. Compare and contrast fatty acid oxidation and synthesis with respect to

(a) site of the process.

(b) acyl carrier.

(c) reductants and oxidants.

(d) stereochemistry of the intemediates.

(e) direction of synthesis or degradation.

(f) organization of the enzyme system.

See answer

4. *Sources*. For each of the following unsaturated fatty acids, indicate whether the biosynthetic precursor in animals is palmitoleate, oleate, linoleate, or linolenate.

(a) 18:1 cis-Δ^{11}

(b) 18:3 cis-Δ^{6},Δ^{9},Δ^{12}

(c) 20:2 cis-Δ^{11},Δ^{14}

(d) 20:3 cis-Δ^{5},Δ^{8},Δ^{11}

(e) 22:1 cis-Δ^{13}

(f) 22:6 cis-Δ^{4},Δ^{7},Δ^{10},Δ^{13},Δ^{16},Δ^{19}

See answer

5. *Tracing carbons*. Consider a cell extract that actively synthesizes palmitate. Suppose that a fatty acid synthase in this preparation forms one molecule of palmitate in about 5 minutes. A large amount of malonyl CoA labeled with ^{14}C in each carbon of its malonyl unit is suddenly added to this system, and fatty acid synthesis is stopped a minute later by altering the pH. The fatty acids in the supernatant are analyzed for radioactivity. Which carbon atom of the palmitate formed by this system is more radioactive—C-1 or C-14?

See answer

6. *Driven by decarboxylation*. What is the role of decarboxylation in fatty acid synthesis? Name another key reaction in a metabolic pathway that employs this mechanistic motif.

See answer

7. *Kinase surfeit*. Suppose that a promoter mutation leads to the overproduction of protein kinase A in adipose cells. How might fatty acid metabolism be altered by this mutation?

See answer

8. *An unaccepting mutant*. The serine residue in acetyl CoA carboxylase that is the target of the AMP-dependent protein kinase is mutated to alanine. What is a likely consequence of this mutation?

See answer

9. *Blocked assets*. The presence of a fuel molecule in the cytosol does not ensure that it can be effectively used. Give two examples of how impaired transport of metabolites between compartments leads to disease.

See answer

10. *Elegant inversion*. Peroxisomes have an alternative pathway for oxidizing polyunsaturated fatty acids. They contain a hydratase that converts D-3-hydroxyacyl CoA into *trans*-Δ^{2}-enoyl CoA. How can this enzyme be used to oxidize CoAs containing a cis double bond at an even-numbered carbon atom (e.g., the cis-Δ^{12} double bond of linoleate)?

See answer

11. *Covalent catastrophe*. What is a potential disadvantage of having many catalytic sites together on one very long polypeptide chain?

See answer

12. *Missing acyl CoA dehydrogenases*. A number of genetic deficiencies in acyl CoA dehydrogenases have been described. This deficiency presents early in life after a period of fasting. Symptoms include vomiting, lethargy, and sometimes coma. Not only are blood levels of glucose low (hypoglycemia), but starvation-induced ketosis is absent. Provide a biochemical explanation for these last two observations.

See answer

13. *Effects of clofibrate*. High blood levels of triacylglycerides are associated with heart attacks and strokes. Clofibrate, a drug that increases the activity of peroxisomes, is sometimes used to treat patients with such a condition. What is the biochemical basis for this treatment?

See answer

14. *A different kind of enzyme*. Figure 22.27 shows the response of acetyl CoA carboxylase to varying amounts of citrate. Explain this effect in light of the allosteric effects that citrate has on the enzyme. Predict the effects of increasing concentrations of palmitoyl CoA.

See answer

Mechanism Problems

15. *Variation on a theme*. Thiolase is homologous in structure to the condensing enzyme. On the basis of this observation, propose a mechanism for the cleavage of 3-ketoacyl CoA by CoA.

See answer

16. *Two plus three to make four*. Propose a reaction mechanism for the condensation of an acetyl unit with a malonyl unit to form an acetoacetyl unit in fatty acid synthesis.

See answer

Chapter Integration Problems

17. *Ill-advised diet*. Suppose that, for some bizarre reason, you decided to exist on a diet of whale and seal blubber, exclusively.

(a) How would lack of carbohydrates affect your ability to utilize fats?

(b) What would your breath smell like?

(c) One of your best friends, after trying unsuccessfully to convince you to abandon this diet, makes you promise to consume a healthy dose of odd-chain fatty acids. Does your friend have your best interests at heart? Explain.

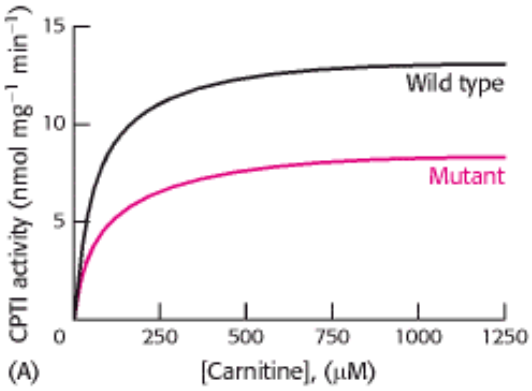
See answer

18. *Fats to glycogen*. An animal is fed stearic acid that is radioactively labeled with [^{14}C]carbon. A liver biopsy reveals the presence of ^{14}C-labeled glycogen. How is this possible in light of the fact that animals cannot convert fats into carbohydrates?

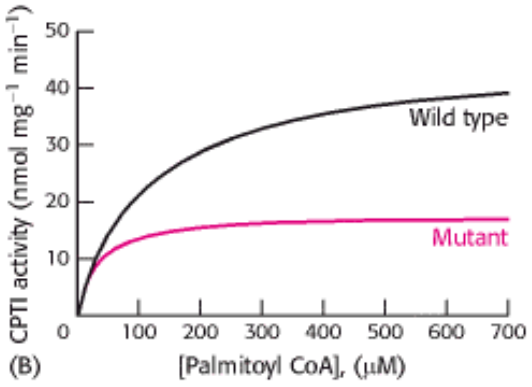
See answer

Data Interpretation Problem

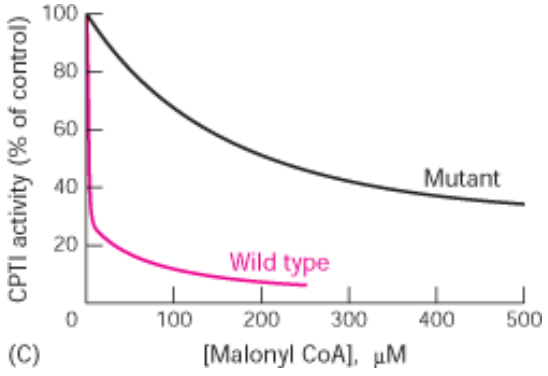
19. *Mutant enzyme*. Carnitine palmitoyl transferase I (CPTI) catalyzes the conversion of long-chain acyl CoA into acyl carnitine, a prerequisite for transport into mitochondria and subsequent degradation. A mutant enzyme was constructed with a single amino acid change at position 3 of glutamic acid for alanine. Figures A through C show data from studies performed to identify the effect of the mutation [data from J. Shi, H. Zhu, D. N. Arvidson, and G. J. Wodegiorgis. *J. Biol. Chem.* 274(1999): 9421–9426].



(a) What is the effect of the mutation on enzyme activity when the concentration of carnitine is varied? What are the K_M and V_{max} values for the wild-type and mutant enzymes?



(b) What is the effect when the experiment is repeated with varying concentrations of palmitoyl CoA? What are the K_M and V_{max} values for the wild-type and mutant enzymes?



(c) Figure C shows the inhibitory effect of malonyl CoA on the wild-type and mutant enzymes. Which enzyme is more sensitive to malonyl CoA inhibition?

(d) Suppose that palmitoyl CoA = 100 μM, carnitine = 100 μM, and malonyl CoA = 10 μM. Under these conditions, what is the most prominent effect of the mutation on the properties of the enzyme?

(e) What can you conclude about the role of glutamate 3 in carnitine acyltransferase I function?

See answer

Media Problem

20. *E pluribus unum*. The storage of chemical energy in the form of fatty acids requires carbon building blocks in the form of acetyl-CoA and energy in the form of ATP and NADPH. Using the **Conceptual Insights** overview of carbohydrate and fatty acid metabolism, estimate how many glucose molecules would be required to form one molecule of palmitate (C_{16}) if glucose were the sole carbon and energy source.

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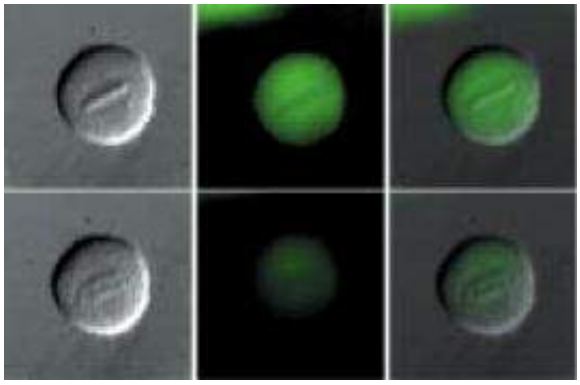
23. Protein Turnover and Amino Acid Catabolism

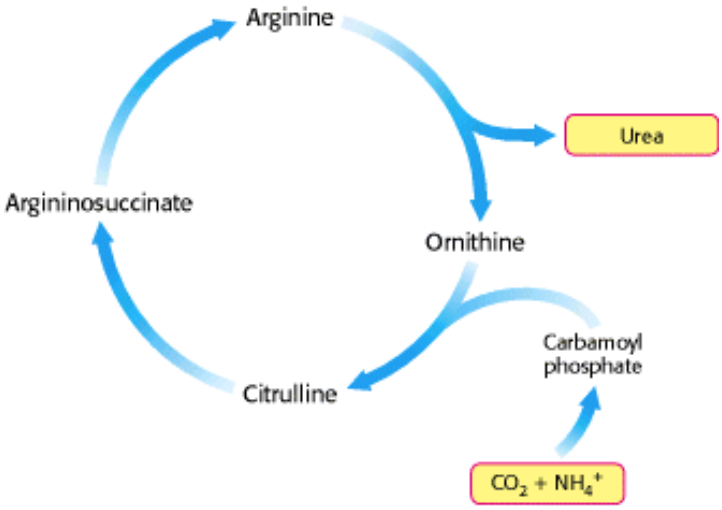
The assembly of new proteins requires a source of amino acids. These building blocks are generated by the digestion of proteins in the intestine and the degradation of proteins within the cell. Many cellular proteins are constantly degraded and resynthesized. To facilitate this recycling, a complex system for the controlled turnover of proteins has evolved. Damaged or unneeded proteins are marked for destruction by the covalent attachment of chains of a small protein, *ubiquitin.* Polyubiquitinated proteins are subsequently degraded by a large, ATP-dependent complex called the *proteasome.*

The primary uses of amino acids are as building blocks for protein and peptide synthesis and as a source of nitrogen for the synthesis of other amino acids and other nitrogenous compounds such as nucleotide bases. Amino acids in excess of those needed for biosynthesis cannot be stored, in contrast with fatty acids and glucose, nor are they excreted. Rather, surplus amino acids are used as metabolic fuel. *The α-amino group is removed, and the resulting carbon skeleton is converted into a major metabolic intermediate.* Most of the amino groups of surplus amino acids are converted into urea through the *urea cycle,* whereas their carbon skeletons are transformed into acetyl CoA, acetoacetyl CoA, pyruvate, or

one of the intermediates of the citric acid cycle. Hence, *fatty acids, ketone bodies, and glucose can be formed from amino acids.*

Several coenzymes play key roles in amino acid degradation, foremost among them is *pyridoxal phosphate.* This coenzyme forms Schiff-base intermediates that allow α-amino groups to be shuttled between amino acids and ketoacids. We will consider several genetic errors of amino acid degradation that lead to brain damage and mental retardation unless remedial action is initiated soon after birth. *Phenylketonuria,* which is caused by a block in the conversion of phenylalanine into tyrosine, is readily diagnosed and can be treated by removing phenylalanine from the diet. The study of amino acid metabolism is especially rewarding because it is rich in connections between basic biochemistry and clinical medicine.





Degradation of cyclin B. This important protein in cell cycle regulation is visible as the green areas in the images above (the protein was fused with green fluorescent protein). Cyclin B is prominent during metaphase, but is degraded in anaphase to prevent premature initiation of another cell cycle. A large protease complex called the proteasome digests the protein into amino acids. These are either reused, or further processed by the urea cycle, which removes the nitrogen as urea. [(Above left) Courtesy of Jonathan Pines, University of Cambridge, Wellcome/CRC Institute of Cancer and Developmental Biology.]

23.1. Proteins Are Degraded to Amino Acids

Dietary protein is a vital source of amino acids. Proteins ingested in the diet are digested into amino acids or small peptides that can be absorbed by the intestine and transported in the blood. Another source of amino acids is the degradation of defective or unneeded cellular proteins.

23.1.1. The Digestion and Absorption of Dietary Proteins

Protein digestion begins in the stomach, where the acidic environment favors protein denaturation. Denatured proteins are more accessible as substrates for proteolysis than are native proteins. The primary proteolytic enzyme of the stomach is *pepsin*, a nonspecific protease that, remarkably, is maximally active at pH 2. Thus, pepsin can be active in the highly acidic environment of the stomach, even though other proteins undergo denaturation there.

Protein degradation continues in the lumen of the intestine owing to the activity of proteolytic enzymes secreted by the pancreas. These proteins, introduced in Chapters 9 and 10 (Sections 9.1 and 10.5), are secreted as inactive zymogens and then converted into active enzymes. The battery of enzymes displays a wide array of specificity, and so the substrates are degraded into free amino acids as well as di- and tripeptides. Digestion is further enhanced by proteases, such as aminopeptidase N, that are located in the plasma membrane of the intestinal cells. Aminopeptidases digest proteins from the amino-terminal end. Single amino acids, as well as di- and tripeptides, are transported into the intestinal cells from the lumen and subsequently released into the blood for absorption by other tissues (Figure 23.1).

23.1.2. Cellular Proteins Are Degraded at Different Rates

Protein turnover—the degradation and resynthesis of proteins—takes place constantly in cells. Although some proteins are very stable, many proteins are short lived, particularly those that are important in metabolic regulation. Altering the amounts of these proteins can rapidly change metabolic patterns. In addition, cells have mechanisms for detecting and removing damaged proteins. A significant proportion of newly synthesized protein molecules are defective because of errors in translation. Even proteins that are normal when first synthesized may undergo oxidative damage or be altered in other ways with the passage of time.

The half-lives of proteins range over several orders of magnitude (Table 23.1). Ornithine decarboxylase, at approximately 11 minutes, has one of the shortest half-lives of any mammalian protein. This enzyme participates in the synthesis of polyamines, which are cellular cations essential for growth and differentiation. The life of hemoglobin, on the other hand, is limited only by the life of the red blood cell, and the lens protein, crystallin, by the life of the organism.

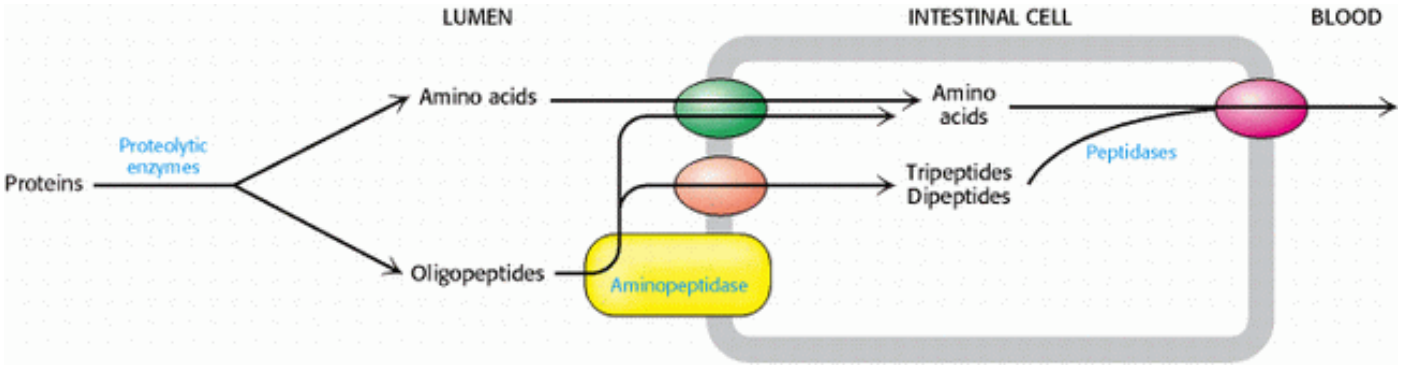


Figure 23.1. Digestion and Absorption of Proteins. Protein digestion is primarily a result of the activity of enzymes secreted by the pancreas. Aminopeptidases associated with the intestinal epithelium further digest proteins. The amino

acids and di- and tripeptides are absorbed into the intestinal cells by specific transporters. Free amino acids are then released into the blood for use by other tissues.

Table 23.1. Dependence of the half-lives of cytosolic yeast proteins on the nature of their amino-terminal residues

Highly stabilizing residues			
($t_{1/2}$ > 20 hours)			
Ala	Cys	Gly	Met
Pro	Ser	Thr	Val
Intrinsically destabilizing residues			
($t_{1/2}$ = 2 to 30 minutes)			
Arg	His	Ile	Leu
Lys	Phe	Trp	Tyr
Destabilizing residues after chemical modification			
($t_{1/2}$ = 3 to 30 minutes)			
Asn	Asp	Gln	Glu

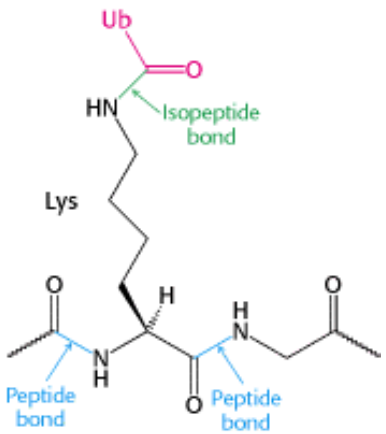
Source: J. W. Tobias, T. E. Schrader, G. Rocap, and A. Varshavsky. *Science* 254(1991):1374.

23.2. Protein Turnover Is Tightly Regulated

How can a cell distinguish proteins that are meant for degradation? *Ubiq-uitin*, a small (8.5-kd) protein present in all eukaryotic cells, is the tag that marks proteins for destruction (Figure 23.2). Ubiquitin is the cellular equivalent of the "black spot" of Robert Louis Stevenson's *Treasure Island:* the signal for death.

23.2.1. Ubiquitin Tags Proteins for Destruction

Ubiquitin is highly conserved in eukaryotes: yeast and human ubiquitin differ at only 3 of 76 residues. The carboxyl-terminal glycine residue of ubiquitin (Ub) becomes covalently attached to the ε-amino groups of several lysine residues on a protein destined to be degraded. The energy for the formation of these *isopeptide bonds* (*iso* because ε- rather than α-amino groups are targeted) comes from ATP hydrolysis.



Three enzymes participate in the attachment of ubiquitin to each protein (Figure 23.3): ubiquitin-activating enzyme, or E1, ubiquitin-conjugating enzyme, or E2, and ubiquitin-protein ligase, or E3. First, the terminal carboxylate group of ubiquitin becomes linked to a sulfhydryl group of E1 by a thioester bond. This ATP-driven reaction is reminiscent of fatty acid activation (Section 22.4.1). An adenylate is linked to the C-terminal carboxylate of ubiquitin with the release of pyrophosphate, and the ubiquitin is transferred to a sulfhydryl group of a key cysteine residue in E1. Second, activated ubiquitin is shuttled to a sulfhydryl group of E2. Finally, E3 catalyzes the transfer of ubiquitin from E2 to an ε-amino group on the target protein.

The attachment of a single molecule of ubiquitin is only a weak signal for degradation. However, the ubiquitination reaction is processive: chains of ubiquitin can be generated by the linkage of the ε-amino group of lysine residue 48 of one ubiquitin molecule to the terminal carboxylate of another. Chains of four or more ubiquitin molecules are particularly effective in signaling degradation (Figure 23.4). The use of chains of ubiquitin molecules may have at least two advantages. First, the ubiquitin molecules interact with one another to form a binding surface distinct from that created by a single ubiquitin molecule. Second, individual ubiquitin molecules can be cleaved off without loss of the degradation signal.

Although most eukaryotes have only one or a small number of distinct E1 enzymes, all eukaryotes have many distinct E2 and E3 enzymes. Moreover, there appears to be only a single family of evolutionarily related E2 proteins but many distinct families of E3 proteins. Although the E3 component provides most of the substrate specificity for ubiquitination, the multiple combinations of the E2–E3 complex allow for more finely tuned substrate discrimination.

What determines whether a protein becomes ubiquitinated? One signal turns out to be unexpectedly simple. *The half-life of a cytosolic protein is determined to a large extent by its amino-terminal residue* (see Table 23.1). This dependency is referred to as the *N-terminal rule.* A yeast protein with methionine at its N terminus typically has a half-life of more than 20 hours, whereas one with arginine at this position has a half-life of about 2 minutes. A highly destabilizing N-terminal residue such as arginine or leucine favors rapid ubiquitination, whereas a stabilizing residue such as methionine or proline does not. E3 enzymes are the readers of N-terminal residues. Other signals thought to identify proteins for degradation include *cyclin destruction boxes*, which are amino acid sequences that mark cell-cycle proteins for destruction, and proteins rich in proline, glutamic acid, serine, and threonine (PEST sequences).


Some pathological conditions vividly illustrate the importance of the regulation of protein turnover. For example, human papilloma virus (HPV) encodes a protein that activates a specific E3 enzyme. The enzyme ubiquitinates the tumor suppressor p53 and other proteins that control DNA repair, which are then destroyed. The activation of this E3 enzyme is observed in more than 90% of cervical carcinomas. Thus, the inappropriate marking of key regulatory proteins for destruction can trigger further events, leading to tumor formation.

23.2.2. The Proteasome Digests the Ubiquitin-Tagged Proteins

If ubiquitin is the mark of death, what is the executioner? A large protease complex called the *proteasome* or the *26S proteasome* digests the ubiquitinated proteins. This ATP-driven multisubunit protease spares ubiq-uitin, which is then recycled. The 26S proteasome is a complex of two components: a 20S proteasome, which contains the catalytic activity, and a 19S regulatory subunit.

The 20S complex is constructed from two copies each of 14 subunits and has a mass of 700 kd (Figure 23.5). All 14 subunits are homologous and adopt the same overall structure. The subunits are arranged in four rings of 7 subunits that stack to form a structure resembling a barrel. The components of the two rings at the ends of the barrel are called the α subunits and those of the two central rings the β subunits. The active sites of the protease are located at the N-termini of certain β subunits on the interior of the barrel—specifically, those β chains having an N-terminal threonine or serine residue. The hydroxyl groups of these amino acids are converted into nucleophiles with the assistance of their own amino groups. These nucleophilic groups then attack the carbonyl groups of peptide bonds and form acyl-enzyme intermediates (Section 9.1.2). The structure of the complex sequesters the proteolytic active sites from potential substrates until they are directed into the barrel. Substrates are degraded in a processive manner without the release of

degradation intermediates, until the substrate is reduced to peptides ranging in length from seven to nine residues.

The 20S proteasome is a sealed barrel. Access to its interior is controlled by a 19S regulatory complex, itself a 700-kd complex made up of 20 subunits. This complex binds to both ends of the 20S proteasome core to form the complete 26S proteasome (Figure 23.6). The 19S subunit binds specifically to polyubiquitin chains. Key components of the 19S complex are six distinct ATPases of the AAA class (*A*TPase *a*ssociated with various cellular *a*ctivities) characterized by a conserved 230 amino acid ATP-binding domain of the P-loop NTPase family. This class of ATPase, found in all kingdoms, is associated with a variety of cell functions including cell-cycle regulation and organelle biogenesis. Although the exact role of the ATPase remains uncertain, ATP hydrolysis may assist the 19S complex to unfold the substrate and induce conformational changes in the 20S proteasome so that the substrate can be passed into the center of the complex. Finally, the 19S subunit also contains an isopeptidase that cleaves off intact ubiquitin molecules. Thus, the ubiquitinization pathway and the proteasome cooperate to degrade unwanted proteins. The ubiquitin is recycled and the peptide products are further degraded by other cellular proteases to yield individual amino acids.

23.2.3. Protein Degradation Can Be Used to Regulate Biological Function

Table 23.2 lists a number of physiological processes that are con trolled at least in part by protein degradation. This control is exerted by dynamically altering the stability and abundance of regulatory proteins. Consider, for example, control of the inflammatory response. A transcription factor called *NF-* **κ*B*** (NF for nuclear factor) initiates the expression of a number of the genes that take part in this response. This important factor is itself activated by the degradation of an inhibitory protein, *I-* **κ*B*.** NF- **κB** is maintained in the cytoplasm in its inactive state by association with I-κB (I for inhibitor). In response to inflammatory signals, I- κ β is phosphorylated at two serine residues, creating an E3 binding site. The binding of E3 leads to the ubiquitination and degradation of I- **κB** and thereby disrupts the inhibitor's association with NF- **κB**. The liberated transcription factor migrates to the nucleus to stimulate transcription of the target genes. The NF- **κB**-I- **κB** system illustrates the interplay of several key regulatory motifs: receptor-mediated signal transduction, phosphorylation, compartmentalization, controlled and specific degradation, and selective gene expression.

23.2.4. The Ubiquitin Pathway and the Proteasome Have Prokaryotic Counterparts

Both the ubiquitin pathway and the proteasome appear to be pres- ent in all eukaryotes. Homologs of the proteasome are found in prokaryotes, although the physiological roles of these homologs have not been well established. The proteasomes of some archaea are quite similar in overall structure to their eukaryotic counterparts and similarly have 28 subunits (Figure 23.7). In the archaeal proteasome, however, all α subunits and all β subunits are identical; in eukaryotes, each α or β subunit is one of seven different isoforms. This specialization provides distinct substrate specificity.

Ubiquitin, however, has not been found in prokaryotes. Indeed, the high level of sequence similarity between the human and yeast proteins suggests that ubiquitin, in its present form, diverged relatively recently in evolutionary terms. Ubiquitin's molecular ancestors were recently identified in prokaryotes. Remarkably, these proteins take part not in protein modification but in coenzyme biosynthesis (Figure 23.8). The biosynthesis of thiamine (Section 8.6.1) begins with a sulfide ion derived from cysteine. This sulfide is added to the C-terminal carboxylate of the protein ThiS, which had been activated as an acyl adenylate. The activation of ThiS and the addition of sulfide are catalyzed by the enzyme ThiF. Human E1 includes two tandem regions of 160 amino acids that are 28% identical in amino acid sequence with a region of ThiF from *E. coli.* The evolutionary relationships between these two pathways were cemented by the determination of the three-dimensional structure of ThiS, which revealed a structure very similar to that of ubiquitin, despite being only 14% identical in amino acid sequence (Figure 23.9). Thus, a eukaryotic system for protein modification evolved from a preexisting prokaryotic pathway for coenzyme biosynthesis.

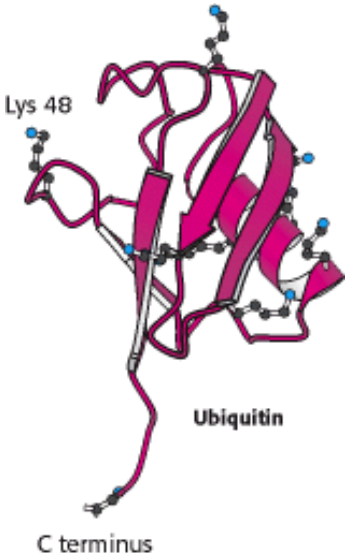


Figure 23.2. Ubiquitin. The structure of ubiquitin reveals an extended carboxyl terminus that is activated and linked to other proteins. Lysine residues also are shown, including lysine 48, the major site for linking additional ubiquitin molecules.

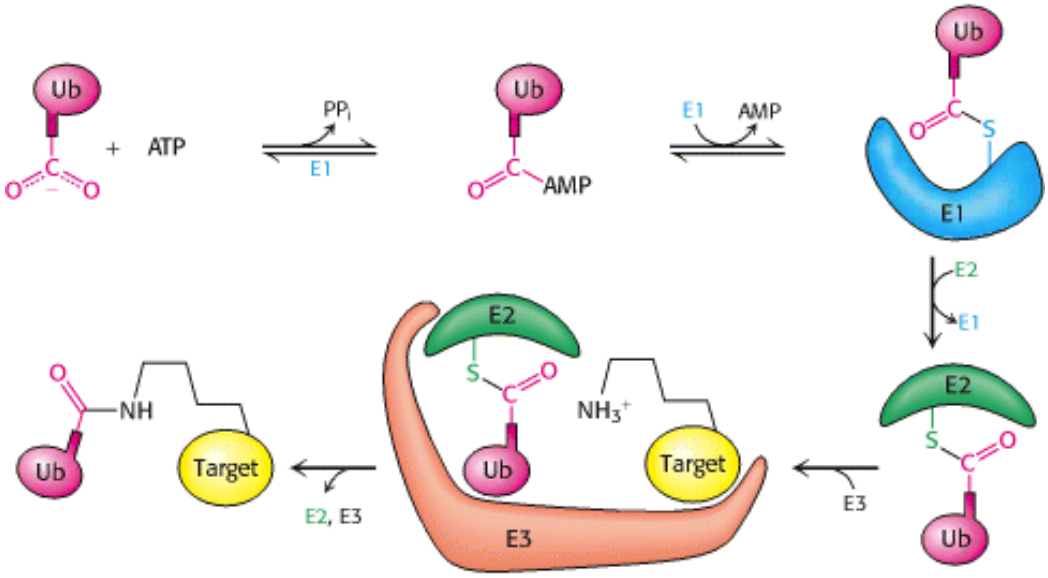


Figure 23.3. Ubiquitin Conjugation. The ubiquitin-activating enzyme E1 adenylates ubiquitin (Ub) and transfers the ubiquitin to one of its own cysteine residues. Ubiquitin is then transferred to a cysteine residue in the ubiquitin-conjugating enzyme E2. Finally, the ubiquitin-protein ligase E3 transfers the ubiquitin to a lysine residue onthe target protein.

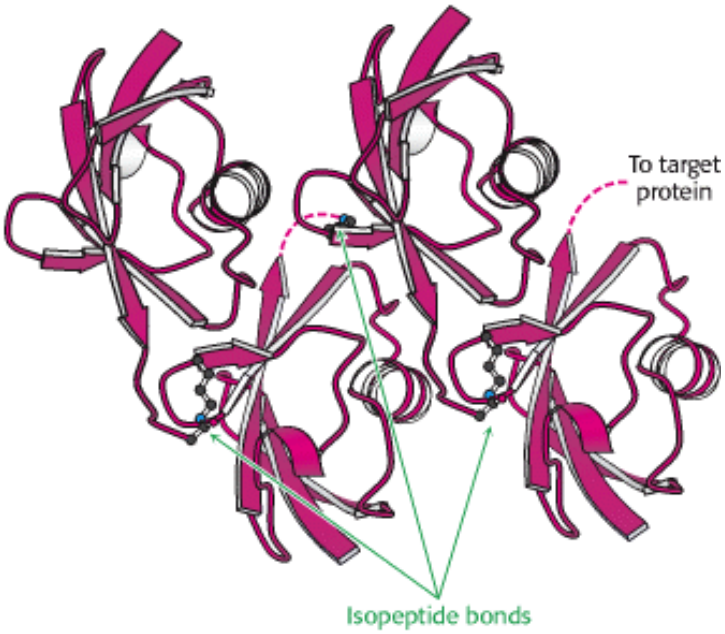


Figure 23.4. Tetra Ubiquitin. Four ubiquitin molecules are linked by isopeptide bonds. This unit is the primary signal for degradation when linked to a target protein.

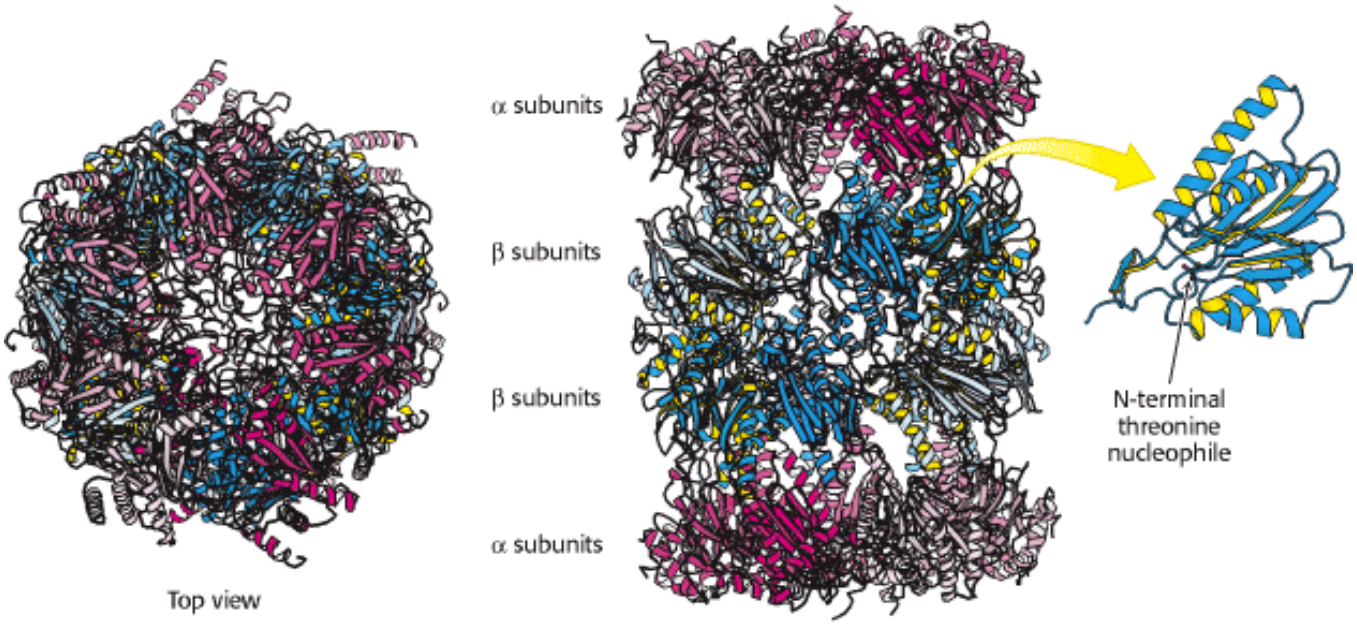


Figure 23.5. 20S Proteasome. The 20S proteasome comprises 28 homologous subunits (α, red; β, blue), arranged in four rings of 7 subunits each. Some of the β subunits (highlighted in yellow) include protease active sites at the amino termini. The top view shows the approximate seven-fold symmetry of the structure.

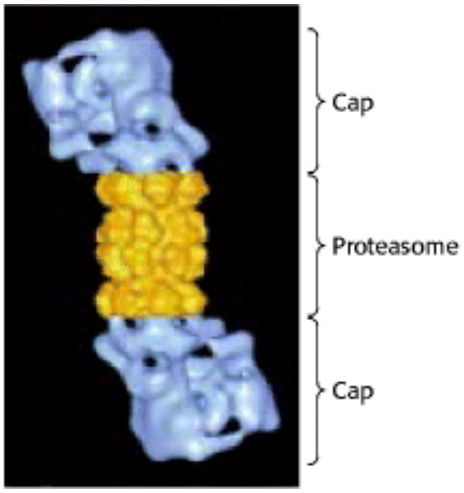
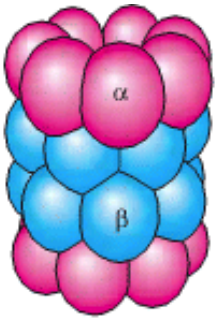


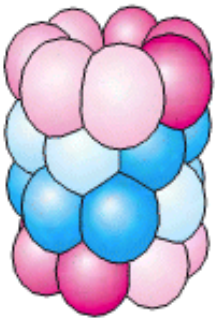
Figure 23.6. 26S Proteasome. A 19S cap is attached to each end of the 20S catalytic unit. [From W. Baumeister, J. Walz, F. Zuhl, and E. Seemuller. *Cell* 92(1998):367. Courtesy of Dr. Wolfgang Baumeister.]

Table 23.2. Processes regulated by protein degradation

Gene transcription
Cell-cycle progression
Organ formation
Circadian rhythms
Inflammatory response
Tumor suppression
Cholesterol metabolism
Antigen processing



Archaeal proteasome



Eukaryotic proteasome

Figure 23.7. Proteasome Evolution. The archaeal proteasome consists of 14 identical α subunits and 14 identical β subunits. In the eukaryotic proteasome, gene duplication and specialization has led to 7 distinct subunits of each type. The overall architecture of the proteasome is conserved.

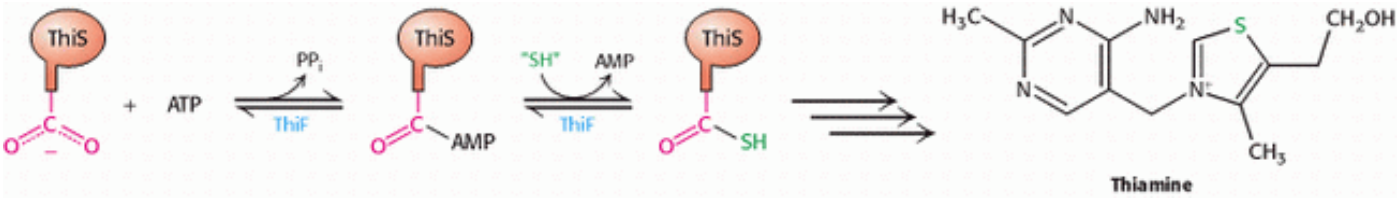


Figure 23.8. Biosynthesis of Thiamine. The biosynthesis of thiamine begins with the addition of sulfide to the carboxyl terminus of the protein ThiS. This protein is activated by adenylation and conjugated in a manner analogous to the first steps in the ubiquitin pathway.

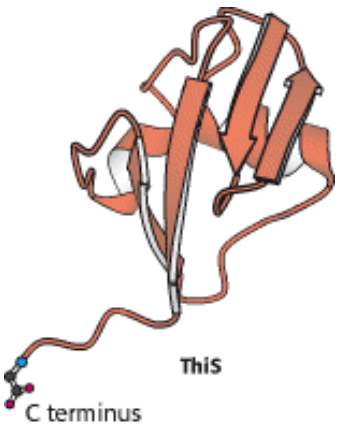


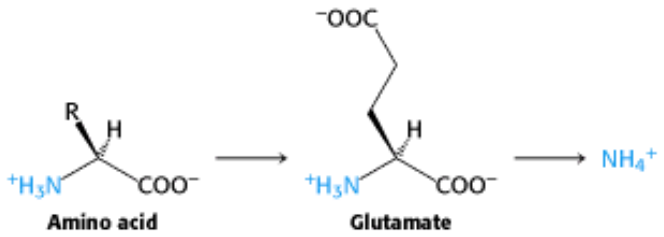
Figure 23.9. Structure of This. The determination of the structure of ThiS revealed it to be structurally similar to ubiquitin despite only 14% sequence identity. This observation suggests that a prokaryotic protein such as ThiS evolved into ubiquitin.

23.3. The First Step in Amino Acid Degradation Is the Removal of Nitrogen

What is the fate of amino acids released on protein digestion or turnover? Any not needed as building blocks are degraded to specific compounds. The major site of amino acid degradation in mammals is the liver. The amino group must be removed, inasmuch as there are no nitrogenous compounds in energy-transduction pathways. The α-ketoacids that result from the deamination of amino acids are metabolized so that the carbon skeletons can enter the metabolic mainstream as precursors to glucose or citric acid cycle intermediates. The fate of the α-amino group will be considered first, followed by that of the carbon skeleton (Section 23.5).

23.3.1. Alpha-Amino Groups Are Converted into Ammonium Ions by the Oxidative Deamination of Glutamate

The α-amino group of many amino acids is transferred to α-ketoglutarate to form *glutamate*, which is then oxidatively deaminated to yield ammonium ion (NH_4^+).



Aminotransferases catalyze the transfer of an α-amino group from an α-amino acid to an α-ketoacid. These enzymes, also called *transaminases*, generally funnel α-amino groups from a variety of amino acids to α-keto-glutarate for conversion into NH_4^+.

Aspartate aminotransferase, one of the most important of these enzymes, catalyzes the transfer of the amino group of aspartate to α-ketoglutarate.

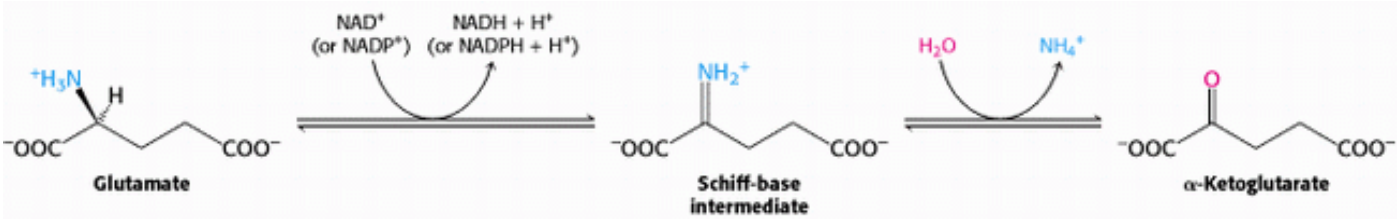
$$\text{Aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{glutamate}$$

Alanine aminotransferase catalyzes the transfer of the amino group of alanine to α-ketoglutarate.

$$\text{Alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{glutamate}$$

These transamination reactions are reversible and can thus be used to synthesize amino acids from α-ketoacids, as we shall see in Chapter 24.

The nitrogen atom that is transferred to α-ketoglutarate in the transamination reaction is converted into free ammonium ion by oxidative deamination. This reaction is catalyzed by *glutamate dehydrogenase.* This enzyme is unusual in being able to utilize either NAD^+ or $NADP^+$, at least in some species. The reaction proceeds by dehydrogenation of the C-N bond, followed by hydrolysis of the resulting Schiff base.



The equilibrium for this reaction favors glutamate; the reaction is driven by the consumption of ammonia. Glutamate dehydrogenase is located in mitochondria, as are some of the other enzymes required for the production of urea. This compartmentalization sequesters free ammonia, which is toxic.

In vertebrates, the activity of glutamate dehydrogenase is allosterically regulated. The enzyme consists of six identical subunits. Guanosine triphosphate and adenosine triphosphate are allosteric inhibitors, whereas guanosine diphosphate and adenosine diphosphate are allosteric activators. Hence, *a lowering of the energy charge accelerates the oxidation of amino acids.*

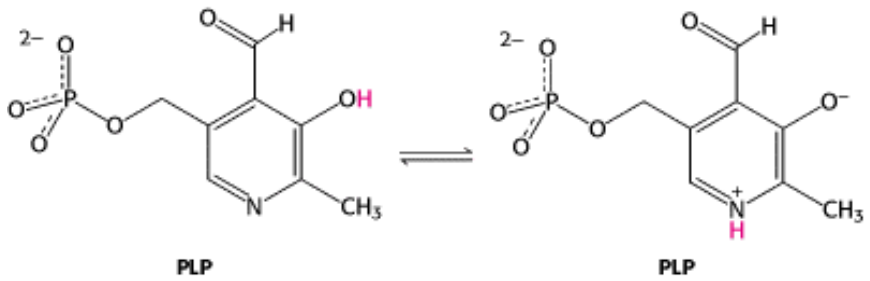
The sum of the reactions catalyzed by aminotransferases and glutamate dehydrogenase is

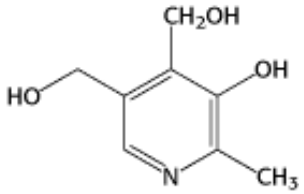
$$\underset{\text{(or NADP}^+\text{)}}{\alpha\text{-Amino acid} + NAD^+} \rightleftharpoons \alpha\text{-ketoacid} + NH_4^+ + \underset{\text{(or NADPH)}}{NADH} + H^+$$

In most terrestrial vertebrates, NH_4^+ is converted into urea, which is excreted.

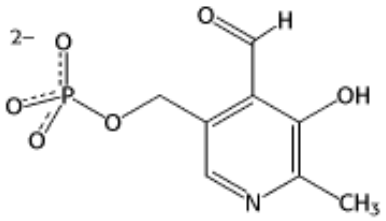
23.3.2. Pyridoxal Phosphate Forms Schiff-Base Intermediates in Aminotransferases

All aminotransferases contain the prosthetic group *pyridoxal phosphate* (PLP), which is derived from *pyridoxine (vitamin* B_6*).* Pyridoxal phosphate includes a pyridine ring that is slightly basic as well as a phenolic hydroxyl group that is slightly acidic. Thus, pyridoxal phosphate derivatives can form stable tautomeric forms in which the pyridine nitrogen atom is protonated and, hence, positively charged while the hydroxyl group is deprotonated, forming a phenolate.



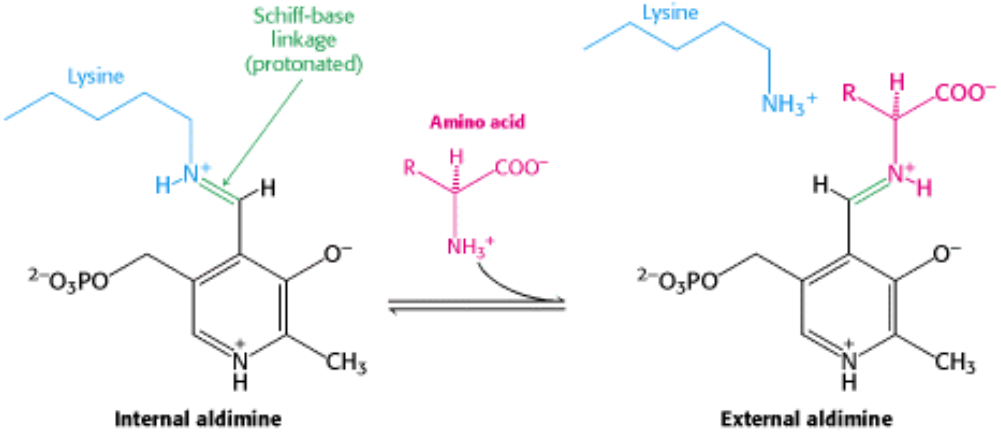


Pyridoxine (Vitamin B_6)



Pyridoxal phosphate (PLP)

The most important functional group on PLP is the aldehyde. This group allows PLP to form covalent Schiff-base intermediates with amino acid substrates. Indeed, even in the absence of substrate, the aldehyde group of PLP usually forms a Schiff-base linkage with the ε-amino group of a specific lysine residue of the enzyme. A new Schiff-base linkage is formed on addition of an amino acid substrate. These Schiff-base linkages are often protonated, with the positive charge stabilized by interaction with the negatively charged phenolate group of PLP.



The α -amino group of the amino acid substrate displaces the ε-amino group of the active-site lysine residue. In other words, an internal aldimine becomes an external aldimine. The amino acid-PLP Schiff base that is formed remains tightly bound to the enzyme by multiple noncovalent interactions.

The Schiff base between the amino acid substrate and PLP, the external *aldimine,* loses a proton from the α-carbon atom of the amino acid to form a *quinonoid* intermediate (Figure 23.10).

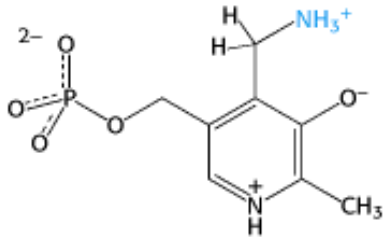
The negative charge that is left on the amino acid is stabilized by delocalization into the pyridinium ring. Reprotonation of this intermediate at the aldehyde carbon atom yields a *ketimine.* The ketimine is then hydrolyzed to an α-ketoacid and *pyridoxamine phosphate (PMP).* These steps constitute half of the transamination reaction.

$$\text{Amino acid}_1 + \text{E-PLP} \rightleftharpoons \alpha\text{-ketoacid}_1 + \text{E-PMP}$$

The second half takes place by the reverse of the preceding pathway. A second α-ketoacid reacts with the enzyme-pyridoxamine phosphate complex (E-PMP) to yield a second amino acid and regenerate the enzyme-pyridoxal phosphate

complex (E-PLP).

$$\alpha\text{-Ketoacid}_2 + \text{E-PMP} \rightleftharpoons \text{amino acid}_2 + \text{E-PLP}$$



Pyridoxamine phosphate (PMP)

The sum of these partial reactions is

$$\text{Amino acid}_1 + \alpha\text{-ketoacid}_2 \rightleftharpoons \text{amino acid}_2 + \alpha\text{-ketoacid}_1$$

23.3.3. Aspartate Aminotransferase Is a Member of a Large and Versatile Family of Pyridoxal-Dependent Enzymes

The mitochondrial enzyme aspartate aminotransferase provides an especially well studied example of PLP as a coenzyme for transamination reactions (Figure 23.11). The results of X-ray crystallographic studies provided detailed views of how PLP and substrates are bound and confirmed much of the proposed catalytic mechanism. Each of the identical 45-kd subunits of this dimer consists of a large domain and a small one. PLP is bound to the large domain, in a pocket near the subunit interface. In the absence of substrate, the aldehyde group of PLP is in a Schiff-base linkage with lysine 258, as anticipated. Adjacent to the coenzyme's binding site is a conserved arginine residue that interacts with the α-carboxylate group of the substrate, helping to orient the substrate appropriately in the active site. The transamination reaction (see Figure 23.10) requires a base to remove a proton from the α-carbon group of the amino acid and to transfer it to the aldehyde carbon atom of PLP. The lysine amino group that was initially in Schiff-base linkage with PLP appears to serve this role.

Transamination is just one of a wide range of amino acid transformations that are catalyzed by PLP enzymes. The other reactions catalyzed by PLP enzymes at the α-carbon atom of amino acids are decarboxylations, deam-inations, racemizations, and aldol cleavages (Figure 23.12). In addition, PLP enzymes catalyze elimination and replacement reactions at the β-carbon atom (e.g., tryptophan synthetase; Section 24.2.11) and the γ-carbon atom (e.g., cytathionine β-synthase, Section 24.2.9) of amino acid substrates. Three common features of PLP catalysis underlie these diverse reactions.

1. A Schiff base is formed by the amino acid substrate (the amine component) and PLP (the carbonyl component).

2. The protonated form of PLP acts as an *electron sink* to stabilize catalytic intermediates that are negatively charged. Electrons from these intermediates can be transferred into the pyridine ring to neutralize the positive charge on the pyridinium nitrogen. In other words, PLP is an *electrophilic catalyst.*

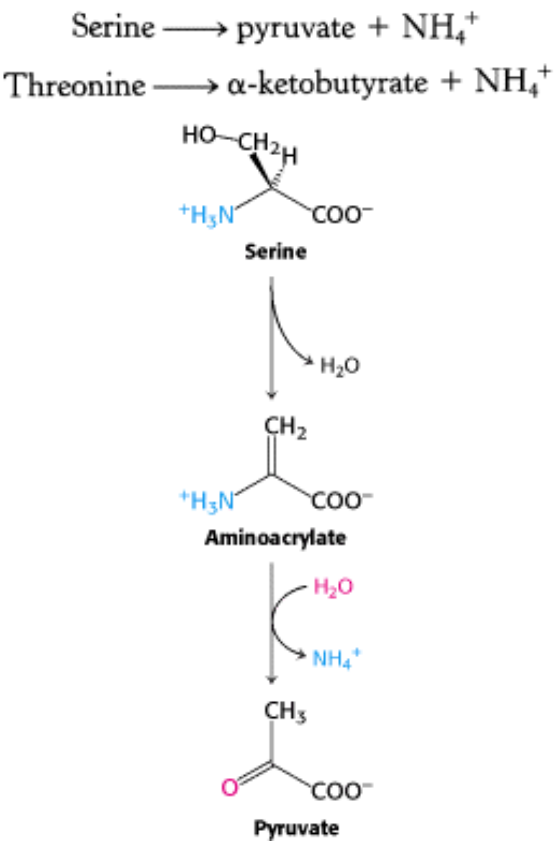
3. The product Schiff base is cleaved at the completion of the reaction.

Many of the enzymes that catalyze these reactions, such as serine hy- droxymethyltransferase, which converts serine into glycine, have the same fold as that of aspartate aminotransferase and are clearly related by divergent evolution. Others, such as tryptophan synthetase, have quite different overall structures. Nonetheless, the active sites of these enzymes are remarkably similar to that of aspartate aminotransferase, revealing the effects of convergent evolution.

How does a particular enzyme selectively favor the cleavage of one of three bonds at the α-carbon atom of an amino acid substrate? An important principle is that *the bond being broken must be perpendicular to the π orbitals of the electron sink* (Figure 23.13). An aminotransferase, for example, achieves this goal by binding the amino acid substrate so that the C_α-H bond is perpendicular to the PLP ring (Figure 23.14). In serine hydroxymethyltransferase, the N-C_α bond is rotated so that the C_α-C_β bond is most nearly perpendicular to the plane of the PLP ring, favoring its cleavage. This means of choosing one of several possible catalytic outcomes is called *stereoelectronic control.*

23.3.4. Serine and Threonine Can Be Directly Deaminated

Although the nitrogen atoms of most amino acids are transferred to α-ketoglutarate before removal, the α-amino groups of serine and threonine can be directly converted into NH_4^+. These direct deaminations are catalyzed by *serine dehydratase* and *threonine dehydratase*, in which PLP is the prosthetic group.



These enzymes are called *dehydratases* because *dehydration precedes deamination.* Serine loses a hydrogen ion from its α-carbon atom and a hydroxide ion group from its β-carbon atom to yield aminoacrylate. This unstable compound reacts with H_2O to give pyruvate and NH_4^+. Thus, the presence of a hydroxyl group attached to the β-carbon atom in each of these amino acids permits the direct deamination.

23.3.5. Peripheral Tissues Transport Nitrogen to the Liver

Most amino acid degradation takes place in tissues other than the liver. For instance, muscle uses amino acids as a source of fuel during prolonged exercise and fasting. How is the nitrogen processed in these other tissues? As in the liver, the first step is the removal of the nitrogen from the amino acid. However, muscle lacks the enzymes of the urea cycle, so the nitrogen must be released in a form that can be absorbed by the liver and converted into urea.

Nitrogen is transported from muscle to the liver in two principal transport forms. Glutamate is formed by transamination reactions, but the nitrogen is then transferred to pyruvate to form alanine, which is released into the blood (Figure 23.15).

The liver takes up the alanine and converts it back into pyruvate by transamination. The pyruvate can be used for gluconeogenesis and the amino group eventually appears as urea. This transport is referred to as the *alanine cycle*. It is reminiscent of the Cori cycle discussed earlier (Section 16.4.2) and again illustrates the ability of the muscle to shift some of its metabolic burden to the liver.

Nitrogen can also be transported as glutamine. Glutamine synthetase (Section 24.1.2) catalyzes the synthesis of glutamine from glutamate and NH_4^+ in an ATP-dependent reaction:

$$NH_4^+ + \text{glutamate} + ATP \xrightarrow{\text{Glutamine synthetase}} \text{glutamine} + ADP + P_i$$

The nitrogens of glutamine can be converted into urea in the liver.

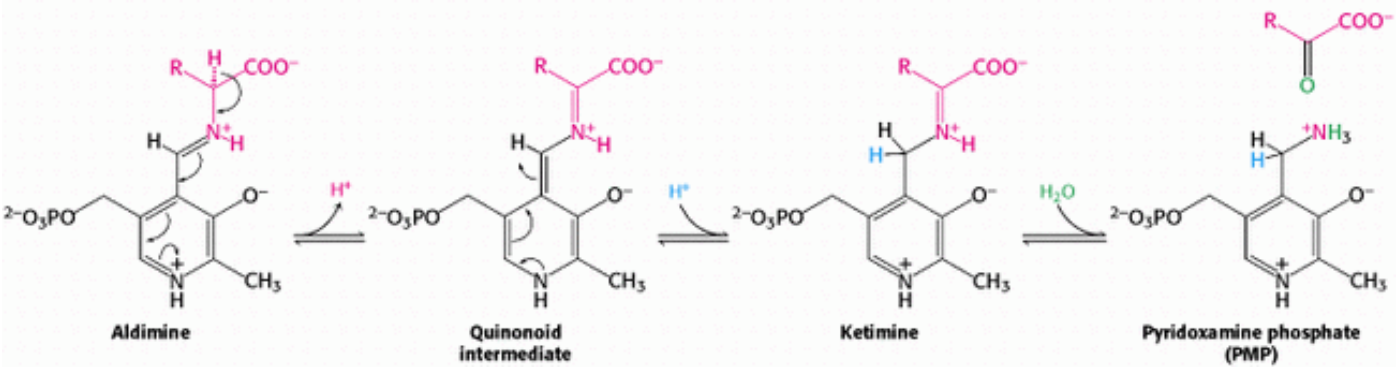


Figure 23.10. Transamination Mechanism. The external aldimine loses a proton to form a quinonoid intermediate. Reprotonation of this intermediate at the aldehyde carbon atom yields a ketimine. This intermediate is hydrolyzed to generate the α-ketoacid product and pyridoxamine phosphate.

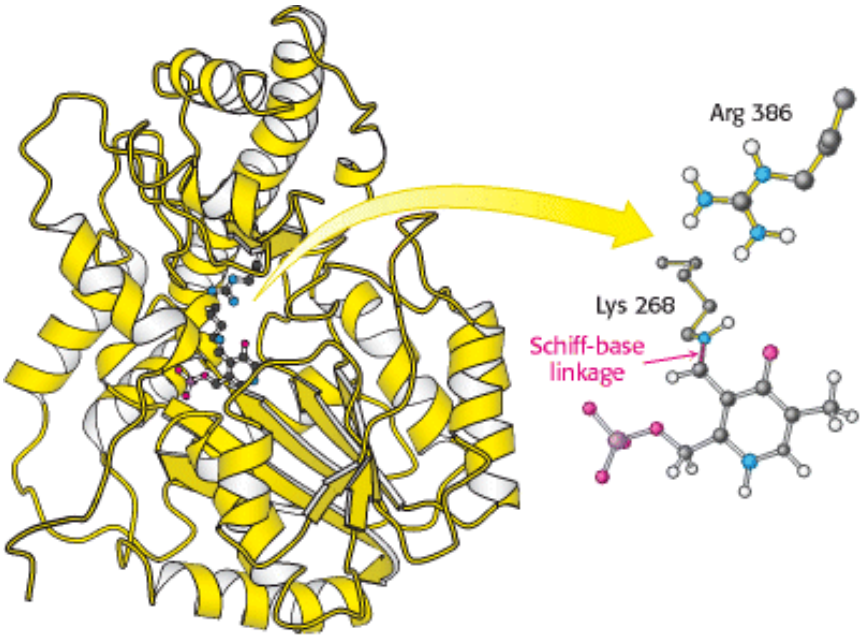


Figure 23.11. Aspartate Aminotransferase. The active site of this prototypical PLP-dependent enzyme includes pyridoxal phosphate attached to the enzyme by a Schiff-base linkage with lysine 258. An arginine residue in the active site helps orient substrates by binding to their α-carboxylate groups.

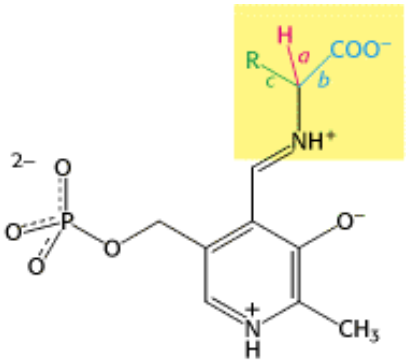


Figure 23.12. Bond Cleavage by PLP Enzymes. Pyridoxal phosphate enzymes labilize one of three bonds at the α-carbon atom of an amino acid substrate. For example, bond *a* is labilized by aminotransferases, bond *b* by decarboxylases, and bond *c* by aldolases (such as threonine aldolases). PLP enzymes also catalyze reactions at the β- and γ-carbon atoms of amino acids.

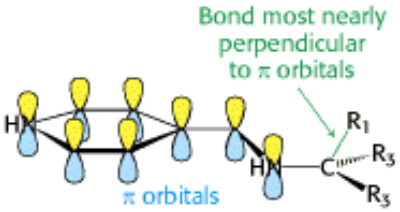


Figure 23.13. Stereoelectronic Effects. The orientation about the NH-Cα bond determines the most favored reaction catalyzed by a pyridoxal phosphate enzyme. The bond that is most nearly perpendicular to the π orbitals of the pyridoxal phosphate electron sink is most easily cleaved.

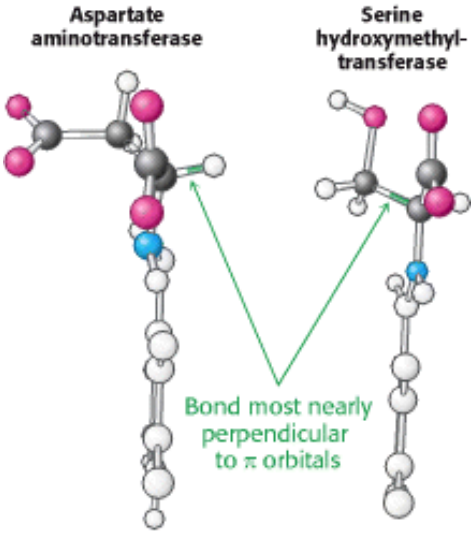


Figure 23.14. Reaction Choice. In aspartate aminotransferase, the Cα-H bond is most nearly perpendicular to the π-orbital system and is cleaved. In serine hydroxymethyltransferase, a small rotation about the N-Cα bond places the Cα-Cβ bond perpendicular to the π system, favoring its cleavage.

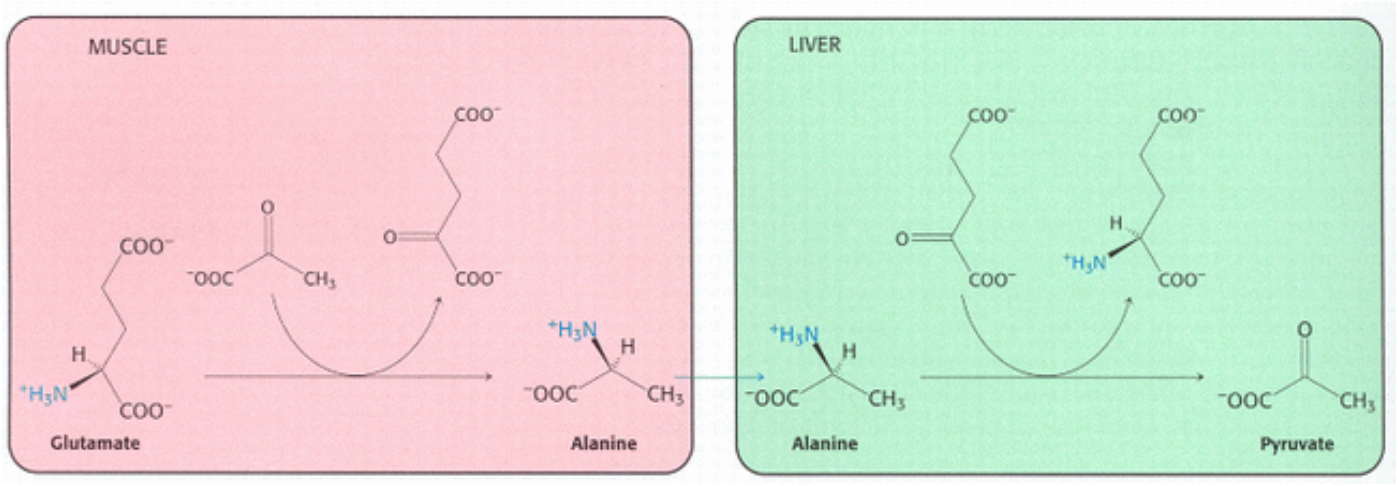
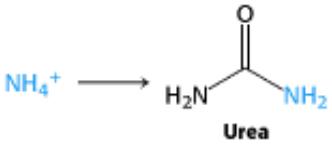


Figure 23.15. The Alanine Cycle. Glutamate in muscle is transaminated to alanine, which is released into the bloodstream. In the liver, alanine is taken up and converted into pyruvate for subsequent metabolism.

23.4. Ammonium Ion Is Converted Into Urea in Most Terrestrial Vertebrates

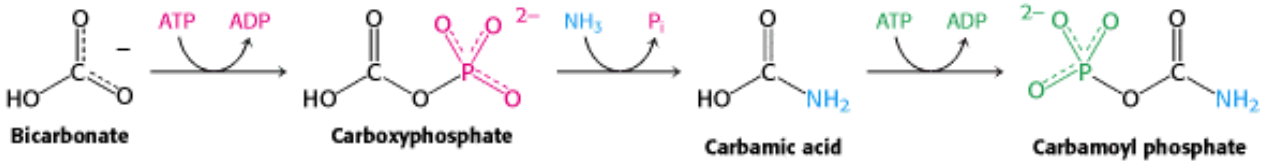
Some of the NH_4^+ formed in the breakdown of amino acids is consumed in the biosynthesis of nitrogen compounds. In most terrestrial vertebrates, the excess NH_4^+ is converted into *urea* and then excreted. Such organisms are referred to as *ureotelic.*

In terrestrial vertebrates, urea is synthesized by the *urea cycle* (Figure 23.16). The urea cycle, proposed by Hans Krebs and Kurt Henseleit in 1932, was the first cyclic metabolic pathway to be discovered. One of the nitrogen atoms of the urea is transferred from an amino acid, aspartate. The other nitrogen atom is derived directly from free NH_4^+, and the carbon atom comes from HCO_3^- (derived by hydration of CO_2; see Section 9.2).



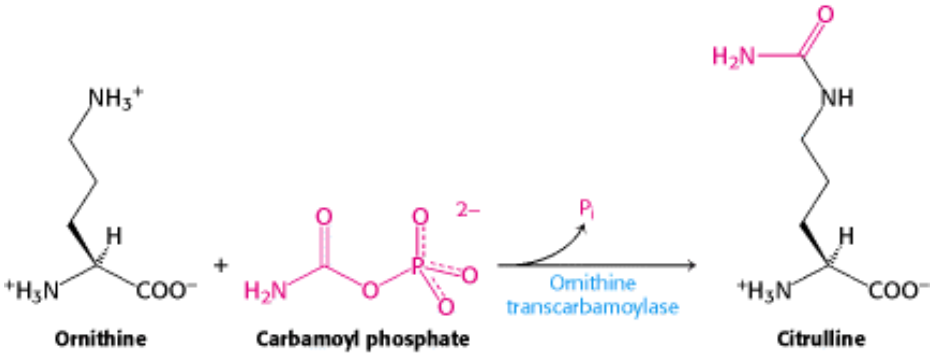
23.4.1. The Urea Cycle Begins with the Formation of Carbamoyl Phosphate

The urea cycle begins with the coupling of free NH_4^+ with HCO_3^- to form carbamoyl phosphate. The synthesis of carbamoyl phosphate, though a simple molecule, comprising three steps, all catalyzed by *carbamoyl phosphate synthetase.*



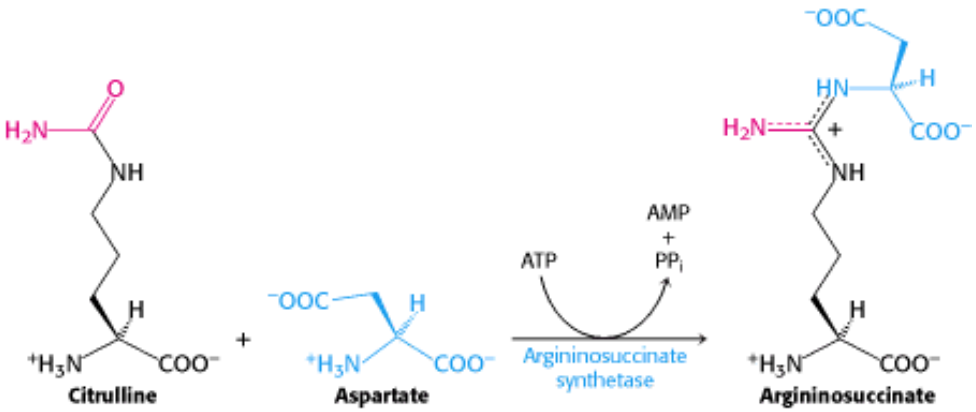
The reaction begins with the phosphorylation of HCO_3^- to form carboxyphosphate, which then reacts with ammonium ion to form carbamic acid. Finally, a second molecule of ATP phosphorylates carbamic acid to carbamoyl phosphate. The structure and mechanism of the fascinating enzyme that catalyzes these reactions will be discussed in Chapter 25. The consumption of two molecules of ATP makes this synthesis of carbamoyl phosphate essentially irreversible. The mammalian enzyme requires N-*acetyl-glutamate* for activity, as will be discussed shortly.

The carbamoyl group of carbamoyl phosphate, which has a high transfer potential because of its anhydride bond, is transferred to *ornithine* to form *citrulline,* in a reaction catalyzed by *ornithine transcarbamoylase.*

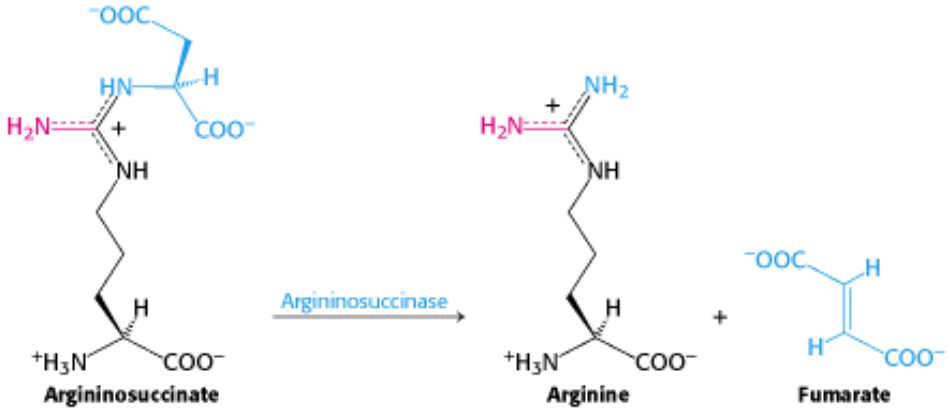


Ornithine and citrulline are amino acids, but they are not used as building blocks of proteins. The formation of NH_4^+ by glutamate dehydrogenase, its incorporation into carbamoyl phosphate, and the subsequent synthesis of citrulline take place in the mitochondrial matrix. In contrast, the next three reactions of the urea cycle, which lead to the formation of urea, take place in the cytosol.

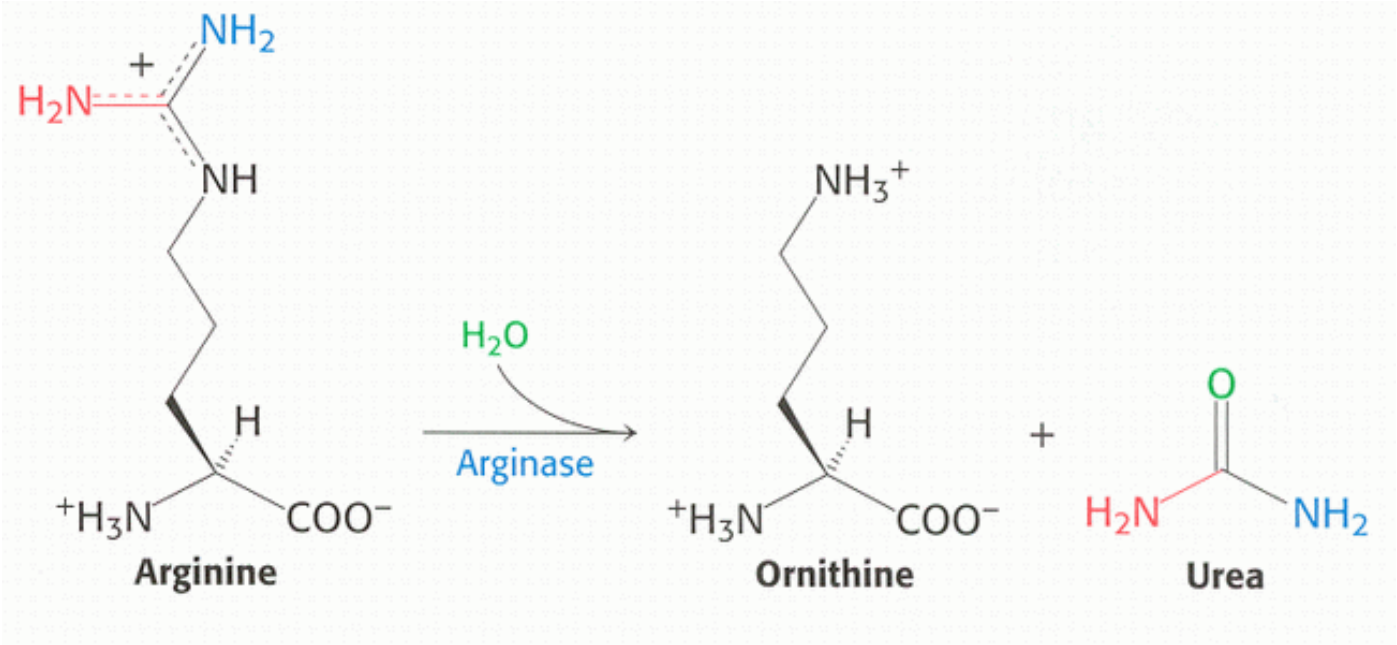
Citrulline is transported to the cytoplasm where it condenses with aspartate, the donor of the second amino group of urea. This synthesis of *argininosuccinate,* catalyzed by *argininosuccinate synthetase,* is driven by the cleavage of ATP into AMP and pyrophosphate and by the subsequent hydrolysis of pyrophosphate.



Argininosuccinase cleaves argininosuccinate into *arginine* and *fumarate.* Thus, the carbon skeleton of aspartate is preserved in the form of fumarate.



Finally, arginine is hydrolyzed to generate urea and ornithine in a reaction catalyzed by *arginase*. Ornithine is then transported back into the mitochondrion to begin another cycle. The urea is excreted. Indeed, human beings excrete about 10 kg (22 pounds) of urea per year.



23.4.2. The Urea Cycle Is Linked to the Citric Acid Cycle

The stoichiometry of urea synthesis is

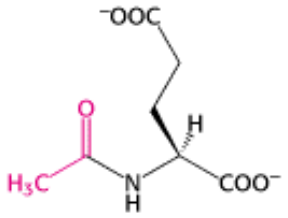
$$CO_2 + NH_4^+ + 3\,ATP + aspartate + 2\,H_2O \longrightarrow urea + 2\,ADP + 2\,P_i + AMP + PP_i + fumarate$$

Pyrophosphate is rapidly hydrolyzed, and so the equivalent of four molecules of ATP are consumed in these reactions to synthesize one molecule of urea. *The synthesis of fumarate by the urea cycle is important because it links the urea cycle and the citric acid cycle* (Figure 23.17). Fumarate is hydrated to malate, which is in turn oxidized to oxaloacetate. Oxaloacetate has several possible fates: (1) transamination to aspartate, (2) conversion into glucose by the gluconeogenic pathway, (3) condensation with acetyl CoA to form citrate, or (4) conversion into pyruvate.

23.4.3. The Evolution of the Urea Cycle

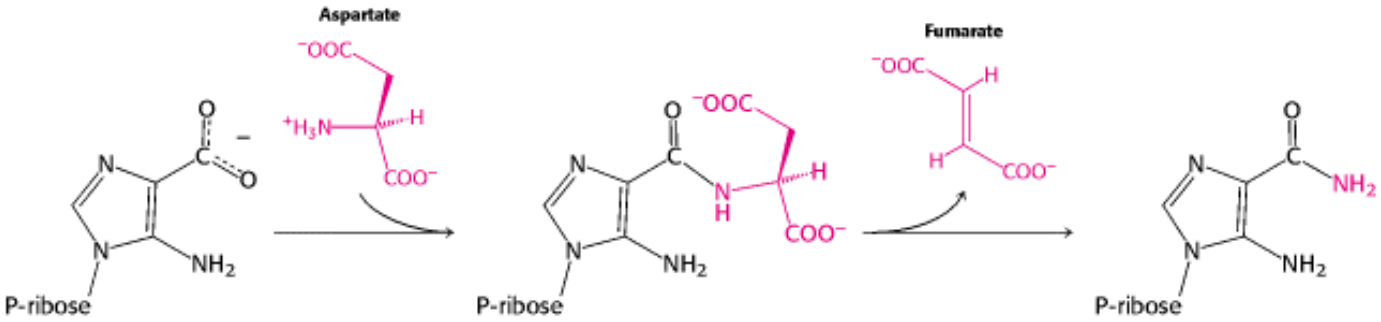
We have previously encountered carbamoyl phosphate as a sub- strate for aspartate transcarbamoylase, the enzyme that catalyzes the first step in pyrimidine biosynthesis (Section 10.1). Carbamoyl phosphate synthetase

generates carbamoyl phosphate for both this pathway and the urea cycle. In mammals, two distinct carbamoyl phosphate synthetase isozymes are present. As discussed earlier, the mitochondrial enzyme uses NH_4^+ as the nitrogen source, as is appropriate for its role in the urea cycle. In pyrimidine biosynthesis, carbamoyl phosphate synthetase differs in two important ways (Section 25.1.1). First, this enzyme utilizes glutamine as a nitrogen source. The side chain amide is hydrolyzed within one domain of the enzyme and the ammonia generated moves through a tunnel in the enzyme to react with carboxyphosphate. Second, this enzyme is part of a large polypeptide called CAD that comprises three distinct enzymes: *c*arbamoyl phosphate synthetase, *a*spartate transcarbamoylase, and *d*ihydroorotase, all of which catalyze steps in pyrimidine biosynthesis (Section 25.1). Interestingly, the domain in which glutamine hydrolysis takes place is largely preserved in the urea-cycle enzyme, although that domain is catalytically inactive. This site binds *N*-acetylglutamate, an allosteric activator of the enzyme. *N*-Acetylglutamate is synthesized only if free amino acids are present, an indication that any ammonia generated must be disposed of. *A catalytic site in one isozyme has been adapted to act as an allosteric site in another isozyme having a different physiological role.*



***N*-Acetylglutamate**

What about the other enzymes in the urea cycle? Ornithine transcarbamoylase is homologous to aspartate transcarbamoylase and the structures of their catalytic subunits are quite similar (Figure 23.18). Thus, two consecutive steps in the pyrimidine biosynthetic pathway were adapted for urea synthesis. The next step in the urea cycle is the addition of aspartate to citrulline to form argininosuccinate, and the subsequent step is the removal of fumarate. These two steps together accomplish the net addition of an amino group to citrulline to form arginine. Remarkably, these steps are analogous to two consecutive steps in the purine biosynthetic pathway (Section 25.2.3).

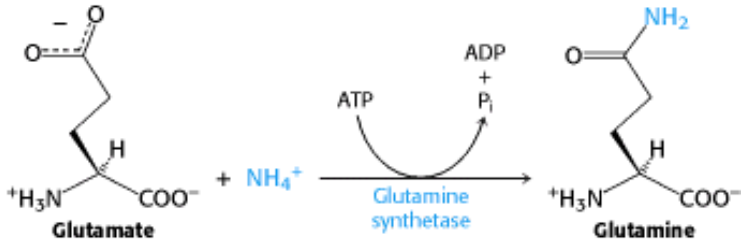


The enzymes that catalyze these steps are homologous to argininosuccinate synthetase and argininosuccinase, respectively. Thus, four of the five enzymes in the urea cycle were adapted from enzymes taking part in nucleotide biosynthesis. The remaining enzyme, arginase, appears to be an ancient enzyme found in all domains of life.

23.4.4. Inherited Defects of the Urea Cycle Cause Hyperammonemia and Can Lead to Brain Damage

The synthesis of urea in the liver is the major route of removal of NH_4^+. A blockage of carbamoyl phosphate synthesis or of any of the four steps of the urea cycle has devastating consequences because there is no alternative pathway for the synthesis of urea. *All defects in the urea cycle lead to an elevated level of NH_4^+ in the blood (hyperammonemia).* Some of these genetic defects become evident a day or two after birth, when the afflicted infant becomes lethargic and vomits periodically. Coma and irreversible brain damage may soon follow. Why are high levels of NH_4^+ toxic? The answer to this question is not yet known. One possibility is that elevated levels of glutamine, formed

from NH_4^+ and glutamate (Section 23.3.5), produce osmotic effects that lead directly to brain swelling.



Ingenious strategies for coping with deficiencies in urea synthesis have been devised on the basis of a thorough understanding of the underlying biochemistry. Consider, for example, *argininosuccinase deficiency.* This defect can be partly bypassed by *providing a surplus of arginine in the diet and restricting the total protein intake.* In the liver, arginine is split into urea and ornithine, which then reacts with carbamoyl phosphate to form citrulline (Figure 23.19). This urea-cycle intermediate condenses with aspartate to yield argininosuccinate, which is then excreted. Note that two nitrogen atoms—one from carbamoyl phosphate and another from aspartate —are eliminated from the body per molecule of arginine provided in the diet. In essence, *argininosuccinate substitutes for urea in carrying nitrogen out of the body.*

The treatment of *carbamoyl phosphate synthetase deficiency* or *ornithine transcarbamoylase deficiency* illustrates a different strategy for circumventing a metabolic block. Citrulline and argininosuccinate cannot be used to dispose of nitrogen atoms, because their formation is impaired. Under these conditions, excess nitrogen accumulates in glycine and glutamine. The challenge then is to rid the body of these two amino acids, which is accomplished by supplementing a protein-restricted diet with *large amounts of benzoate and phenylacetate.* Benzoate is activated to benzoyl CoA, which reacts with glycine to form hippurate (Figure 23.20). Likewise, phenylacetate is activated to phenylacetyl CoA, which reacts with glutamine to form phenylacetylglutamine. These conjugates substitute for urea in the disposal of nitrogen. Thus, *latent biochemical pathways can be activated to partly bypass a genetic defect.*

23.4.5. Urea Is Not the Only Means of Disposing of Excess Nitrogen

As discussed earlier, most terrestrial vertebrates are ureotelic; they excrete excess nitrogen as urea. However, urea is not the only excretable form of nitrogen. *Ammoniotelic organisms, such as aquatic vertebrates and invertebrates, release nitrogen as* NH_4^+ and rely on the aqueous environment to dilute this toxic substance. Interestingly, lungfish, which are normally ammoniotelic, become ureotelic in time of drought, when they live out of the water.

Both ureotelic and ammoniotelic organisms depend on sufficient water, to varying degrees, for nitrogen excretion. *In contrast, uricotelic organisms, which secrete nitrogen as the purine uric acid, require little water.* Disposal of excess nitrogen as uric acid is especially valuable in animals, such as birds, that produce eggs having impermeable membranes that accumulate waste products. The pathway for nitrogen excretion developed in the course of evolution clearly depends on the habitat of the organism.

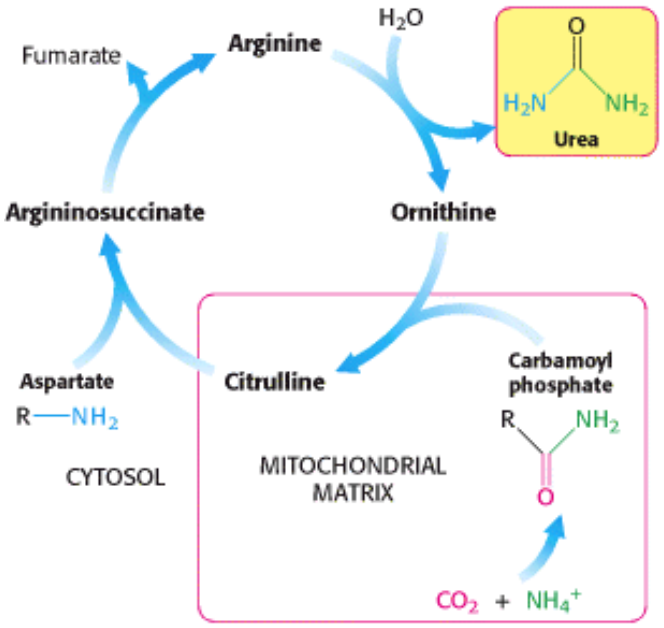


Figure 23.16. The Urea Cycle.

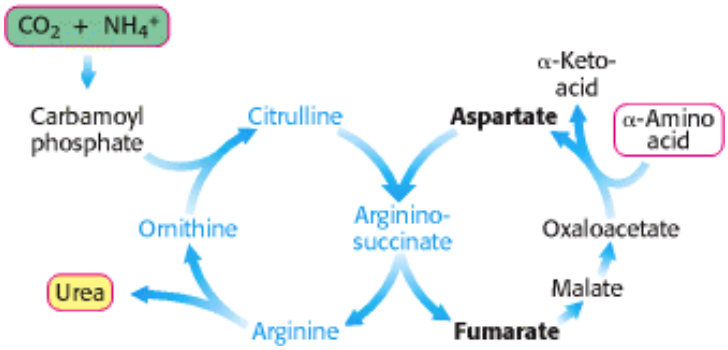


Figure 23.17. Metabolic Integration of Nitrogen Metabolism. The urea cycle, the citric acid cycle, and the transamination of oxaloacetate are linked by fumarate and aspartate.

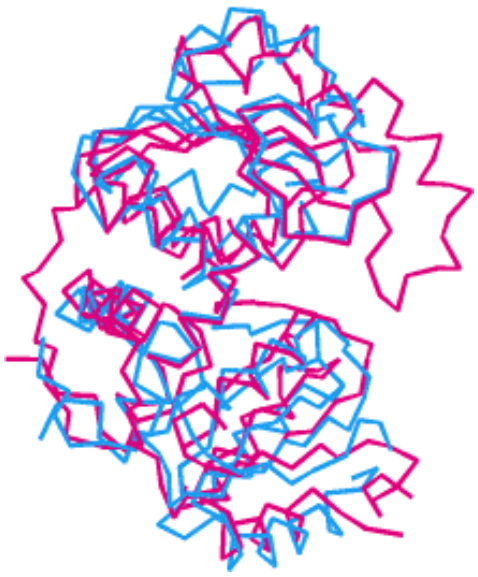


Figure 23.18. Homologous Enzymes. The structure of the catalytic subunit of ornithine transcarbamoylase (blue) is quite similar to that of the catalytic subunit of aspartate transcarbamoylase (red), indicating that these two enzymes are homologs.

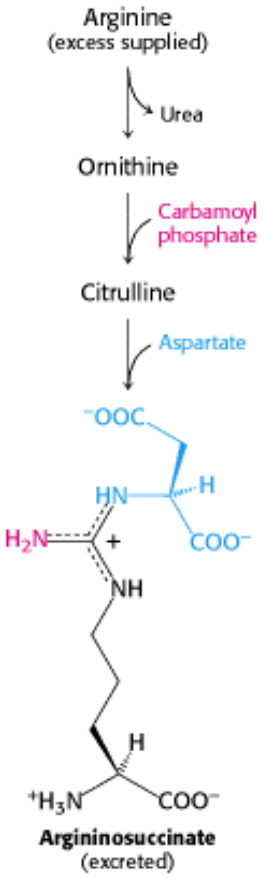


Figure 23.19. Treatment of Argininosuccinase Deficiency. Argininosuccinase deficiency can be managed by supplementing the diet with arginine. Nitrogen is excreted in the form of argininosuccinate.

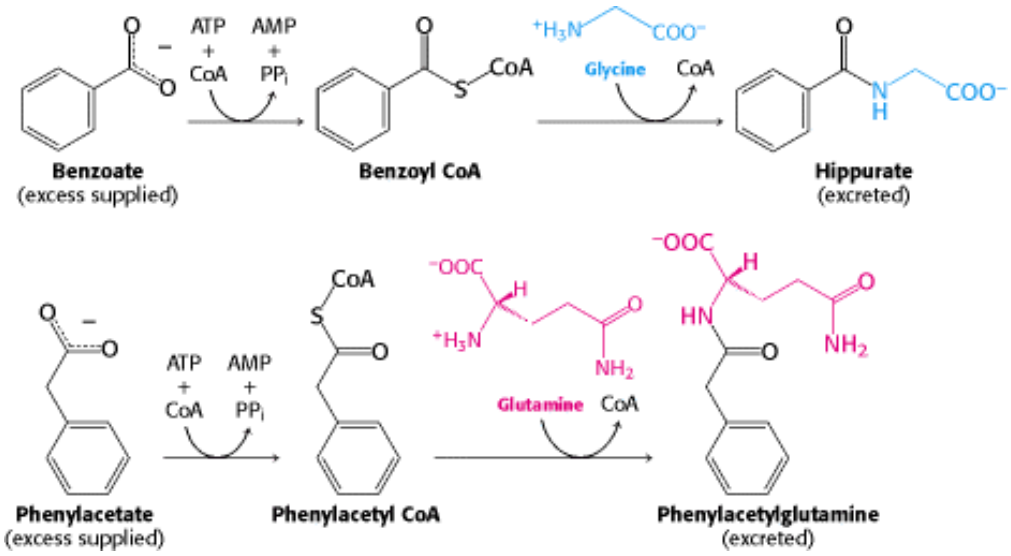


Figure 23.20. Treatment of Carbamoyl Phosphate Synthetase and Ornithine Transcarbamoylase Deficiencies. Both deficiencies can be treated by supplementing the diet with benzoate and phenylacetate. Nitrogen is excreted in the form of hippurate and phenylacetylglutamine.

23.5. Carbon Atoms of Degraded Amino Acids Emerge as Major Metabolic Intermediates

We now turn to the fates of the carbon skeletons of amino acids after the removal of the α-amino group. *The strategy of amino acid degradation is to transform the carbon skeletons into major metabolic intermediates that can be converted into glucose or oxidized by the citric acid cycle.* The conversion pathways range from extremely simple to quite complex. The carbon skeletons of the diverse set of 20 fundamental amino acids are funneled into only seven molecules: *pyruvate, acetyl CoA, acetoacetyl CoA, α-ketoglutarate, succinyl CoA, fumarate,* and *oxaloacetate.* We see here a striking example of the remarkable economy of metabolic conversions, as well as an illustration of the importance of certain metabolites.

Amino acids that are degraded to acetyl CoA or acetoacetyl CoA are termed *ketogenic* amino acids because they can give rise to ketone bodies or fatty acids. Amino acids that are degraded to pyruvate, α-ketoglutarate, succinyl CoA, fumarate, or oxaloacetate are termed *glucogenic* amino acids. The net synthesis of glucose from these amino acids is feasible because these citric acid cycle intermediates and pyruvate can be converted into phosphoenolpyruvate and then into glucose (Section 16.3.2). Recall that mammals lack a pathway for the net synthesis of glucose from acetyl CoA or acetoacetyl CoA.

Of the basic set of 20 amino acids, only leucine and lysine are solely ketogenic (Figure 23.21). Isoleucine, phenylalanine, tryptophan, and tyrosine are both ketogenic and glucogenic. Some of their carbon atoms emerge in acetyl CoA or acetoacetyl CoA, whereas others appear in potential precursors of glucose. The other 14 amino acids are classed as solely glucogenic. This classification is not universally accepted, because different quantitative criteria are applied. Whether an amino acid is regarded as being glucogenic, ketogenic, or both depends partly on the eye of the beholder. We will identify the degradation pathways by the entry point into metabolism.

23.5.1. Pyruvate as an Entry Point into Metabolism

Pyruvate is the entry point of the three-carbon amino acids—alanine, serine, and cysteine—into the metabolic mainstream (Figure 23.22). The transamination of alanine directly yields pyruvate.

$$\text{Alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{glutamate}$$

As mentioned previously (Section 23.3.1), glutamate is then oxidatively deaminated, yielding NH_4^+ and regenerating α-ketoglutarate. The sum of these reactions is

$$\text{Alanine} + \text{NAD(P)}^+ + H_2O \longrightarrow \text{pyruvate} + NH_4^+ + \text{NAD(P)H} + H^+$$

Another simple reaction in the degradation of amino acids is the *deamination of serine to pyruvate* by *serine dehydratase* (Section 23.3.4).

$$\text{Serine} \longrightarrow \text{pyruvate} + NH_4^+$$

Cysteine can be converted into pyruvate by several pathways, with its sulfur atom emerging in H_2S, SCN^-, or SO_3^{2-}.

The carbon atoms of three other amino acids can be converted into pyruvate. *Glycine* can be converted into serine by enzymatic addition of a hydroxymethyl group or it can be cleaved to give CO_2, NH_4^+, and an activated one-carbon unit

(Section 24.2.6). *Threonine* can give rise to pyruvate through the intermediate aminoacetone. Three carbon atoms of *tryptophan* can emerge in alanine, which can be converted into pyruvate.

23.5.2. Oxaloacetate as an Entry Point into Metabolism

Aspartate and asparagine are converted into oxaloacetate, a citric acid cycle intermediate. *Aspartate,* a four-carbon amino acid, is directly *transaminated to oxaloacetate.*

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{glutamate}$$

Asparagine is hydrolyzed by *asparaginase* to NH_4^+ and aspartate, which is then transaminated.

Recall that aspartate can also be converted into *fumarate* by the urea cycle (Section 23.4.2). Fumarate is also a point of entry for half the carbon atoms of tyrosine and phenylalanine, as will be discussed shortly.

23.5.3. Alpha-Ketoglutarate as an Entry Point into Metabolism

The carbon skeletons of several five-carbon amino acids enter the citric acid cycle at α *-ketoglutarate.* These amino acids are first converted into *gluta-mate,* which is then oxidatively deaminated by glutamate dehydrogenase to yield α-ketoglutarate (Figure 23.23).

Histidine is converted into 4-imidazolone 5-propionate (Figure 23.24). The amide bond in the ring of this intermediate is hydrolyzed to the *N*-formimino derivative of glutamate, which is then converted into glutamate by transfer of its formimino group to tetrahydrofolate, a carrier of activated one-carbon units (Section 24.2.6).

Glutamine is hydrolyzed to glutamate and NH_4^+ by *glutaminase. Proline* and *arginine* are each converted into glutamate γ-semialdehyde, which is then oxidized to glutamate (Figure 23.25).

23.5.4. Succinyl Coenzyme A Is a Point of Entry for Several Nonpolar Amino Acids

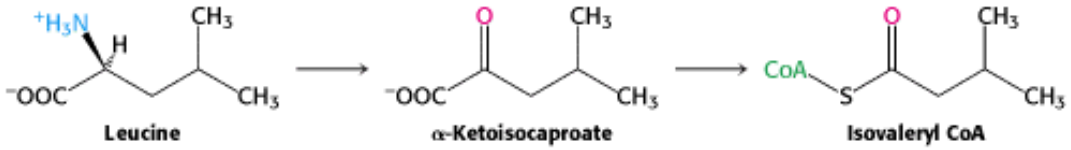
Succinyl CoA is a point of entry for some of the carbon atoms of methio-nine, isoleucine, and valine. Propionyl CoA and then methylmalonyl CoA are intermediates in the breakdown of these three nonpolar amino acids (Figure 23.26). The mechanism for the interconversion of propionyl CoA and methylmalonyl CoA was presented in Section 22.3.3. This pathway from propionyl CoA to succinyl CoA is also used in the oxidation of fatty acids that have an odd number of carbon atoms (Section 22.3.2).

23.5.5. Methionine Degradation Requires the Formation of a Key Methyl Donor, S-Adenosylmethionine

Methionine is converted into succinyl CoA in nine steps (Figure 23.2.7). The first step is the adenylation of methionine to form *S*-adenosylmethionine (SAM), a common methyl donor in the cell (Section 24.2.7). Methyl donation and deadenylation yield homocysteine, which is eventually processed to α-ketobutyrate. The enzyme α-ketoacid dehydrogenase complex oxidatively decarboxylates α-ketobutyrate to propionyl CoA, which is processed to succinyl CoA as described in Section 23.3.3.

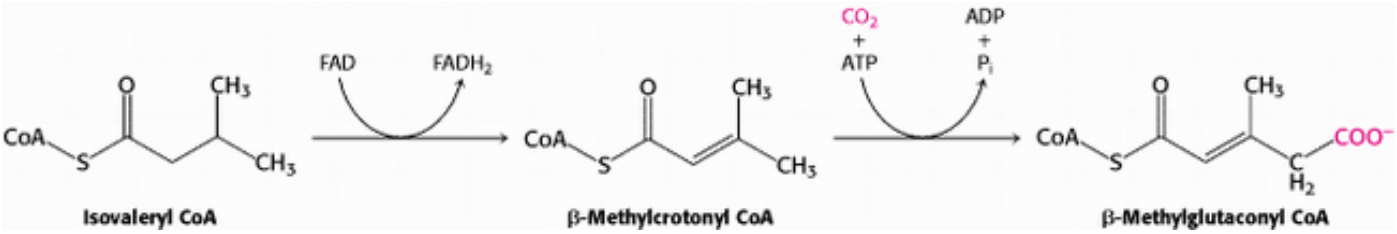
23.5.6. The Branched-Chain Amino Acids Yield Acetyl CoA, Acetoacetate, or Propionyl CoA

The degradation of the branched-chain amino acids employs reactions that we have encountered previously in the citric acid cycle and fatty acid oxidation. Leucine is transaminated to the corresponding α-ketoacid, *α-ketoisocaproate.* This α-ketoacid is *oxidatively decarboxylated to isovaleryl CoA* by the *branched-chain α-ketoacid dehydrogenase complex.*

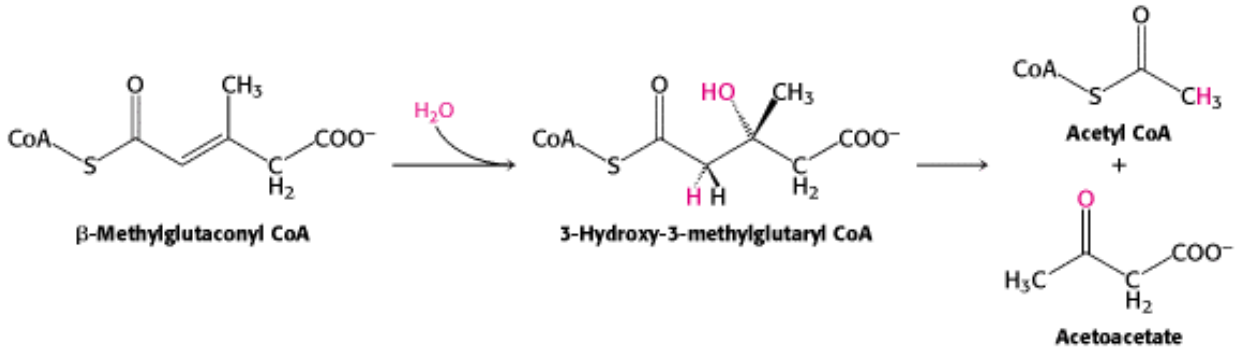


The α-ketoacids of valine and isoleucine, the other two branched-chain aliphatic amino acids, as well as α-ketobutyrate derived from methionine also are substrates. The oxidative decarboxylation of these α-ketoacids is analogous to that of pyruvate to acetyl CoA and of α-ketoglutarate to succinyl CoA. The branched-chain α-ketoacid dehydrogenase, a multienzyme complex, is a homolog of pyruvate dehydrogenase (Section 17.1.1) and α-ketoglutarate dehydrogenase (Section 17.1.6). Indeed, the E3 components of these enzymes, which regenerate the oxidized form of lipoamide, are identical.

The isovaleryl CoA derived from leucine is *dehydrogenated* to yield β-*methylcrotonyl CoA.* This oxidation is catalyzed by *isovaleryl CoA dehydrogenase.* The hydrogen acceptor is FAD, as in the analogous reaction in fatty acid oxidation that is catalyzed by acyl CoA dehydrogenase. β-*Methylglutaconyl CoA* is then formed by the *carboxylation* of β-methylcrotonyl CoA at the expense of the hydrolysis of a molecule of ATP. As might be expected, the carboxylation mechanism of β-methylcrotonyl CoA carboxylase is similar to that of pyruvate carboxylase and acetyl CoA carboxylase.



β-Methylglutaconyl CoA is then *hydrated* to form *3-hydroxy-3-methylglutaryl CoA,* which is cleaved into *acetyl CoA* and *acetoacetate.* This reaction has already been discussed in regard to the formation of ketone bodies from fatty acids (Section 22.3.5).

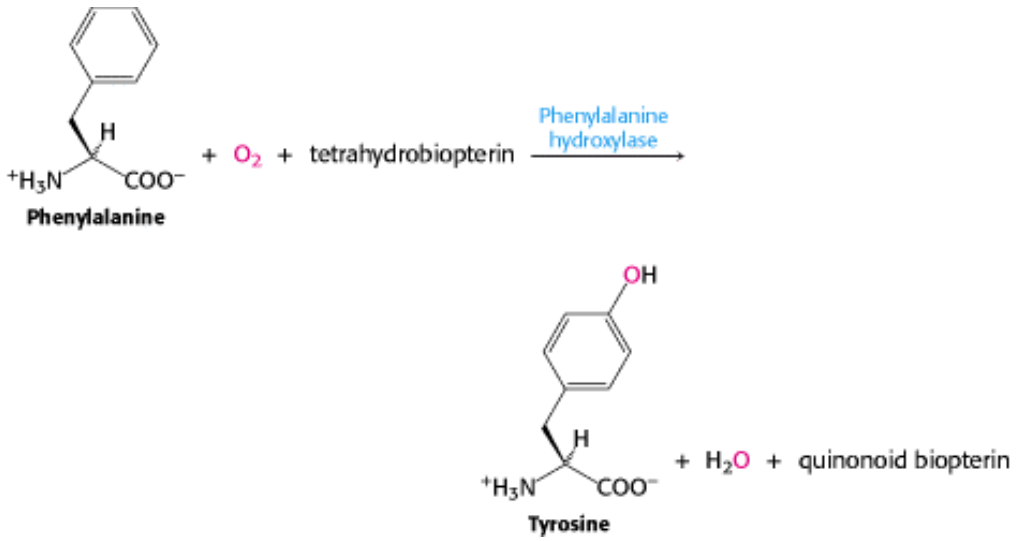


The degradative pathways of valine and isoleucine resemble that of leucine. After transamination and oxidative decarboxylation to yield a CoA derivative, the subsequent reactions are like those of fatty acid oxidation. Isoleucine yields acetyl CoA and propionyl CoA, whereas valine yields CO_2 and propionyl CoA. The degradation of leucine, valine, and isoleucine validate a point made earlier (Chapter 14): the number of reactions in metabolism is large, but the number of *kinds* of reactions is relatively small. The degradation of leucine, valine, and isoleucine provides a striking illustration of the underlying simplicity and elegance of metabolism.

23.5.7. Oxygenases Are Required for the Degradation of Aromatic Amino Acids

The degradation of the aromatic amino acids is not as straightforward as that of the amino acids previously discussed, although the final products—acetoacetate, fumarate, and pyruvate—are common intermediates. For the aromatic amino acids, *molecular oxygen is used to break an aromatic ring.*

The degradation of phenylalanine begins with its hydroxylation to tyrosine, a reaction catalyzed by *phenylalanine hydroxylase.* This enzyme is called a *monooxygenase* (or *mixed-function oxygenase*) because *one atom of* O_2 appears in the product and the other in H_2O.



The reductant here is *tetrahydrobiopterin,* an electron carrier that has not been previously discussed and is derived from the cofactor *biopterin*. Because biopterin is synthesized in the body, it is not a vitamin. Tetrahydrobiopterin is initially formed by the reduction of dihydrobiopterin by NADPH in a reaction catalyzed by *dihydrofolate reductase* (Figure 23.28). NADH reduces the quinonoid form of dihydrobiopterin produced in the hydroxylation of phenylalanine back to tetrahydrobiopterin in a reaction catalyzed by *dihydropteridine reductase.* The sum of the reactions catalyzed by phenylalanine hydroxylase and dihydropteridine reductase is

$$\text{Phenylalanine} + O_2 + \text{NADH} + H^+ \longrightarrow \text{tyrosine} + \text{NAD}^+ + H_2O$$

Note that these reactions can also be used to synthesize tyrosine from phenylalanine.

The next step in the degradation of phenylalanine and tyrosine is the transamination of tyrosine to p-*hydroxyphenylpyruvate* (Figure 23.29). This α-ketoacid then reacts with O_2 to form *homogentisate.* The enzyme catalyzing this complex reaction, p-*hydroxyphenylpyruvate hydroxylase,* is called a *dioxygenase* because *both atoms of* O_2 become incorporated into the product, one on the ring and one in the carboxyl group. The aromatic ring of homogentisate is then cleaved by O_2, which yields 4-maleylacetoacetate. This reaction is catalyzed by *homogentisate oxidase,* another dioxygenase. 4-Maleylacetoacetate is then isomerized to *4-fumarylacetoacetate* by an enzyme that uses glutathione as a cofactor. Finally, 4-fumarylacetoacetate is hydrolyzed to *fumarate* and *acetoacetate.*

Tryptophan degradation requires several oxygenases (Figure 23.30). Tryptophan 2,3-dioxygenase cleaves the pyrrole ring, and kynureinine 3-monooxygenase hydroxylates the remaining benzene ring, a reaction similar to the hydroxylation of phenylalanine to form tyrosine. Alanine is removed and the 3-hydroxyanthranilic acid is cleaved with another dioxygenase and subsequently processed to acetoacetyl CoA (Figure 23.31). *Nearly all cleavages of aromatic rings in*

biological systems are catalyzed by dioxygenases, a class of enzymes discovered by Osamu Hayaishi. The active sites of these enzymes contain iron that is not part of heme or an iron- sulfur cluster.

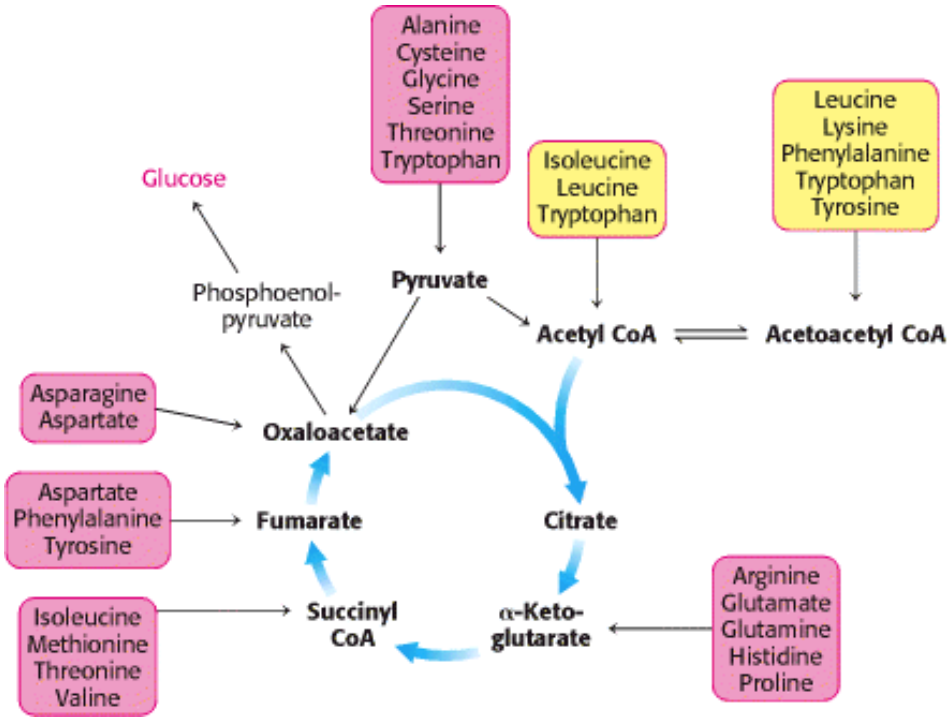


Figure 23.21. Fates of the Carbon Skeletons of Amino Acids. Glucogenic amino acids are shaded red, and ketogenic amino acids are shaded yellow. Most amino acids are both glucogenic and ketogenic.

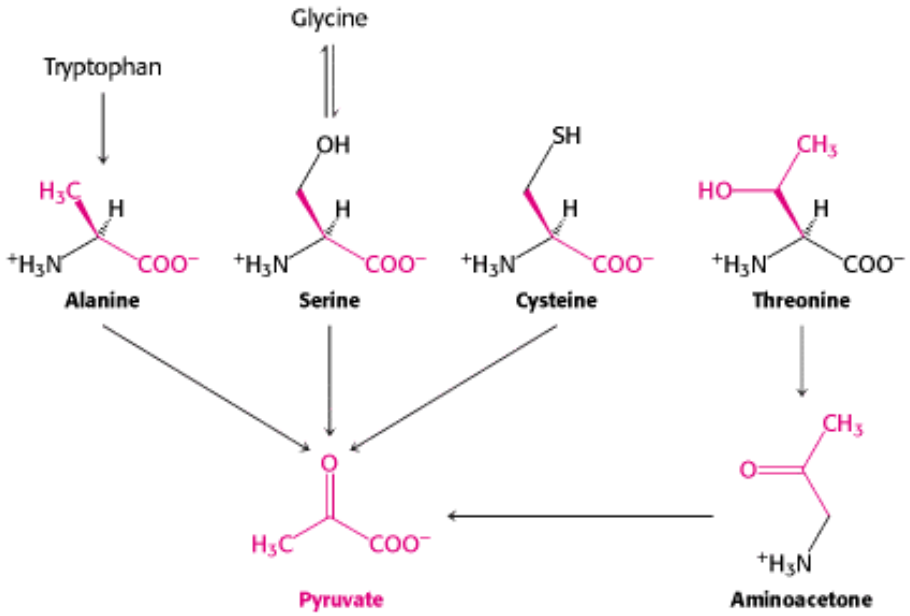


Figure 23.22. Pyruvate Formation from Amino Acids. Pyruvate is the point of entry for alanine, serine, cysteine, glycine, threonine, and tryptophan.

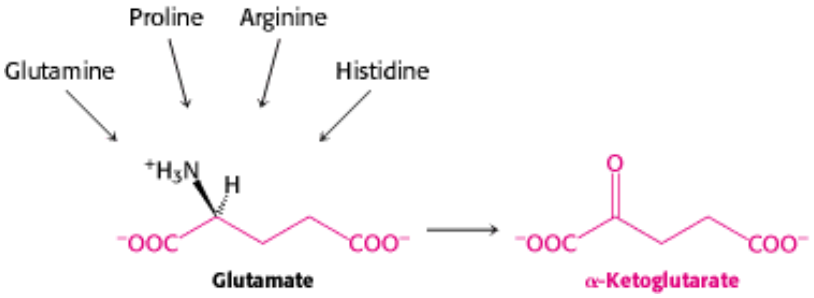


Figure 23.23. α -Ketoglutarate formation from amino acids. α-Ketoglutarate is the point of entry of several five-carbon amino acids that are first converted into glutamate.

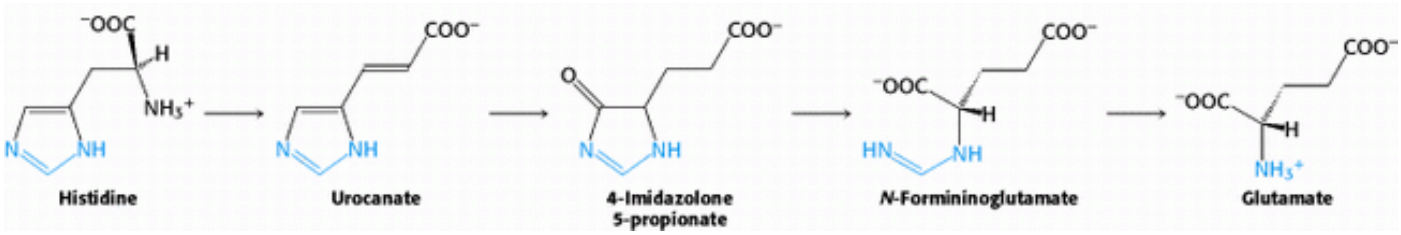


Figure 23.24. Histidine Degradation. Conversion of histidine into glutamate.

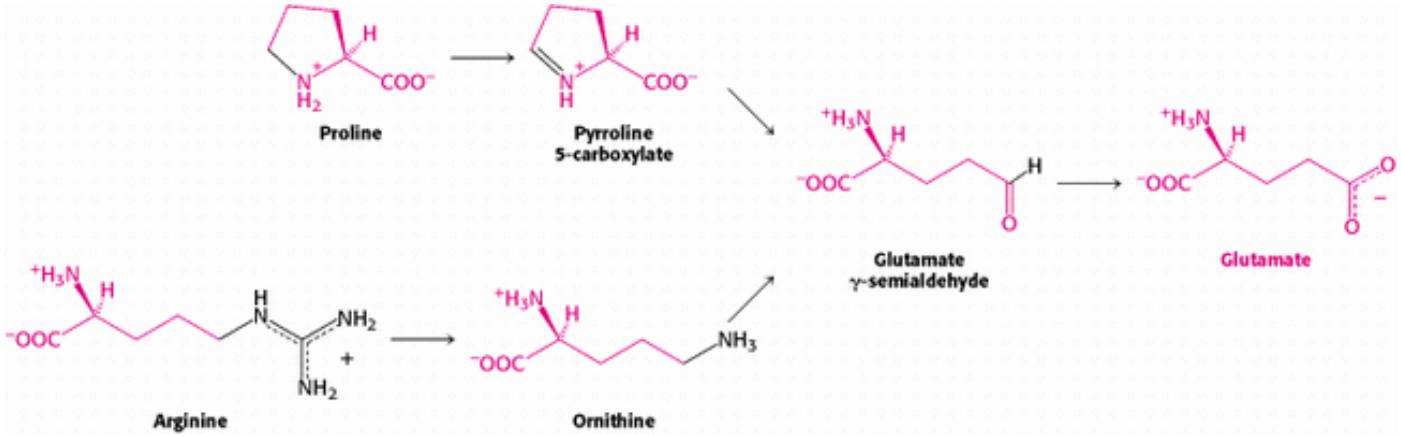


Figure 23.25. Glutamate Formation. Conversion of proline and arginine into glutamate.

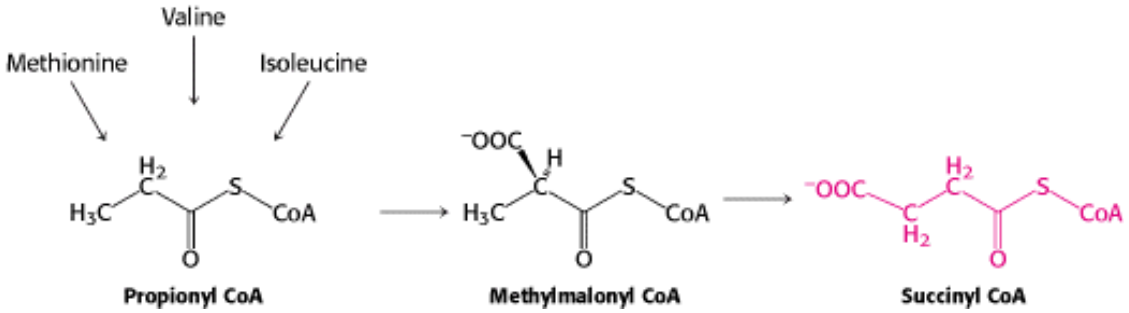


Figure 23.26. Succinyl CoA Formation. Conversion of methionine, isoleucine, and valine into succinyl CoA.

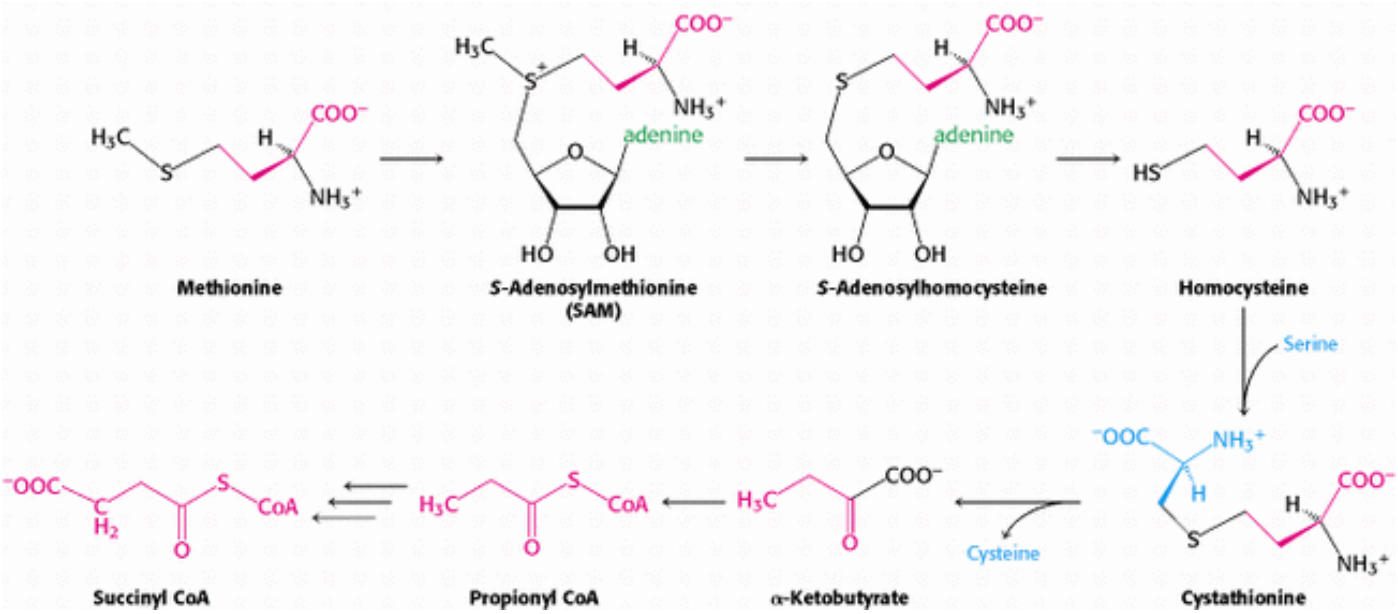


Figure 23.27. Methionine Metabolism. The pathway for the conversion of methionine into succinyl CoA. *S*-Adenosylmethionine, formed along this pathway, is an important molecule for transferring methyl groups.

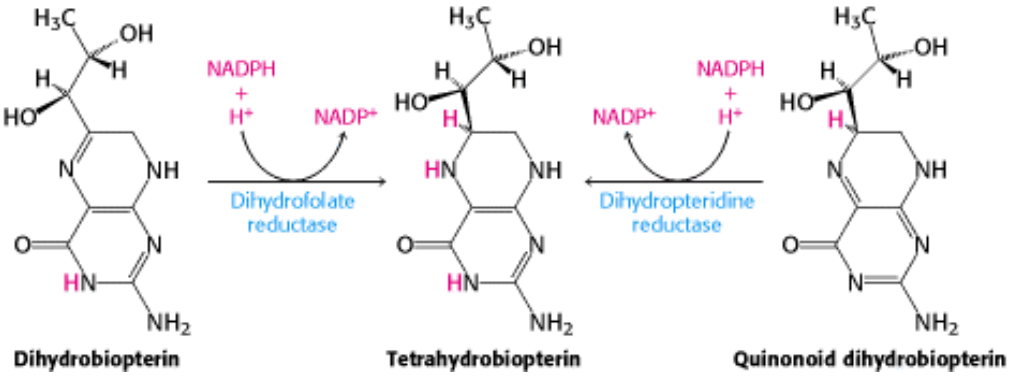


Figure 23.28. Formation of Tetrahydrobiopterin, an Important Electron Carrier. Tetrahydrobiopterin can be formed by the reduction of either of two forms of dihydrobiopterin.

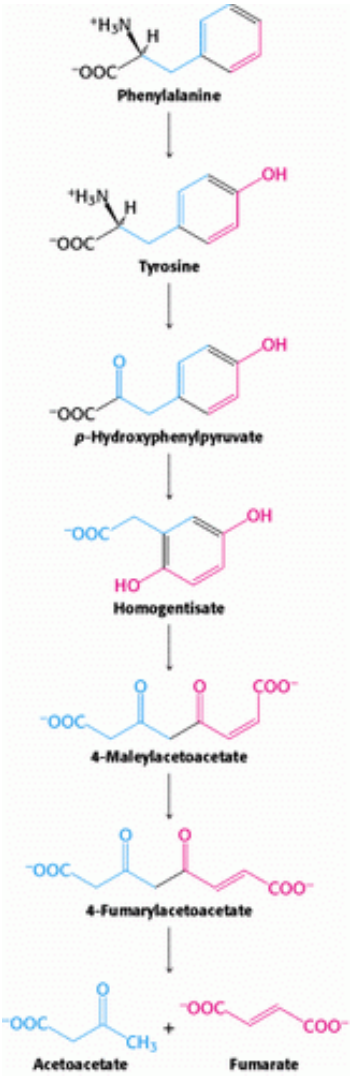


Figure 23.29. Phenylalanine and Tyrosine Degradation. The pathway for the conversion of phenylalanine into acetoacetate and fumarate.

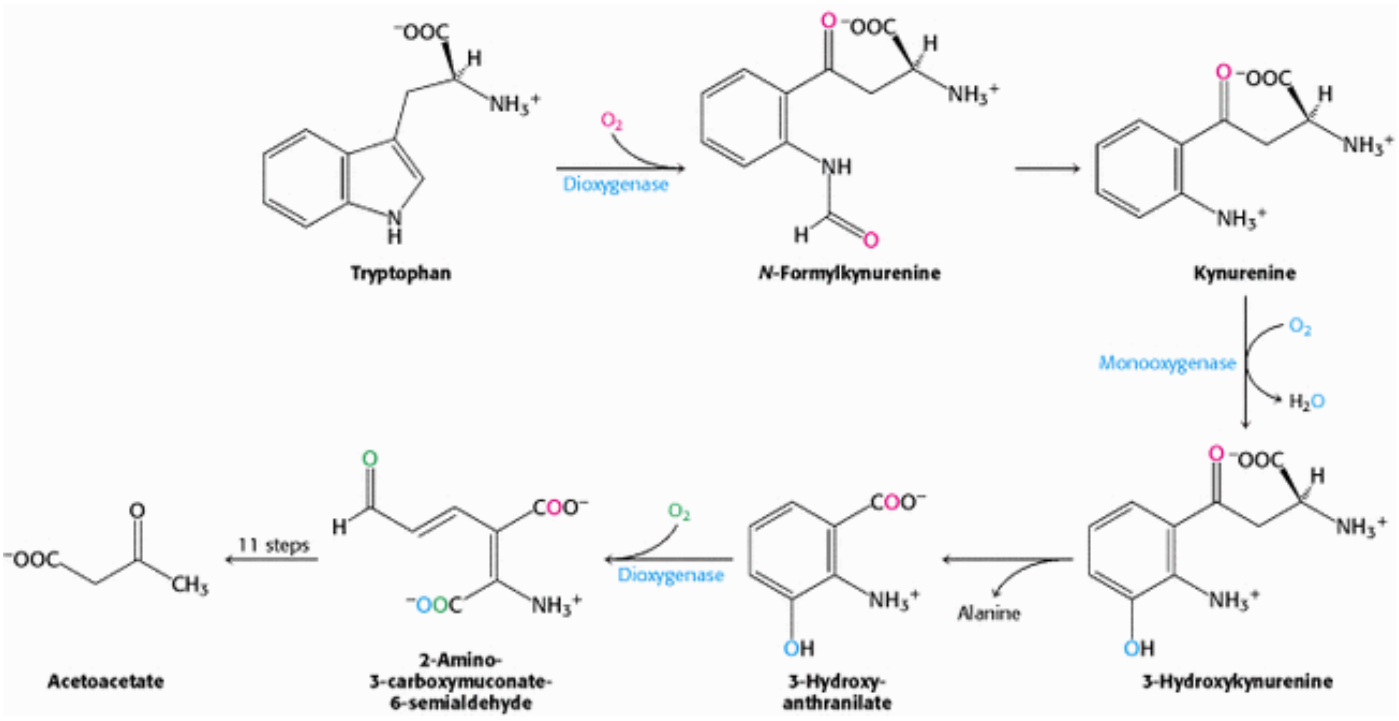


Figure 23.30. Tryptophan Degradation. The pathway for the conversion of tryptophan into alanine and acetoacetate.

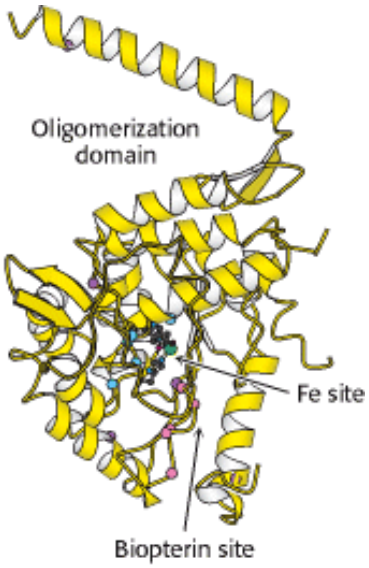
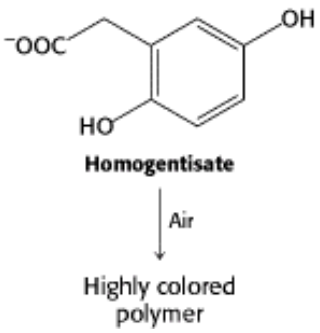


Figure 23.31. Structure of One Subunit of Phenylalanine Hydroxylase. Mutations in the genes encoding this enzyme cause phenylketonuria. More than 200 point mutations have been identified in these genes. The positions of five mutations affecting the active site (blue), the biopterin-binding site (red), and other regions of the protein (purple) are indicated as colored spheres.

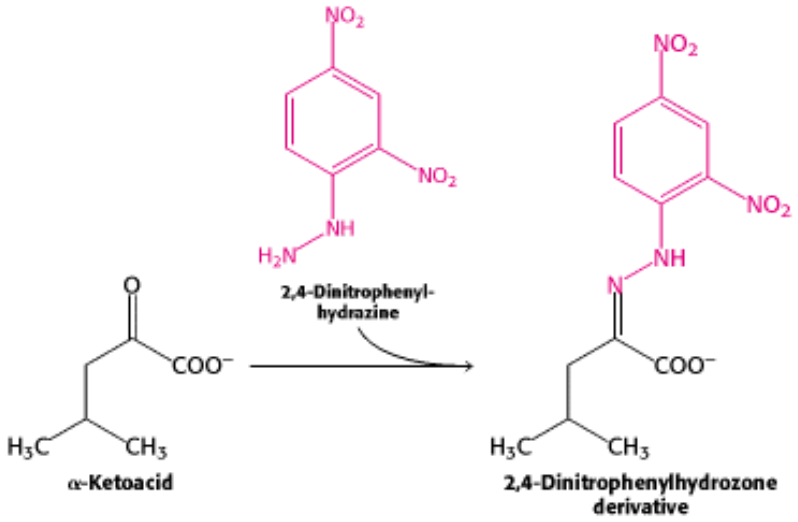
23.6. Inborn Errors of Metabolism Can Disrupt Amino Acid Degradation

Errors in amino acid metabolism provided some of the first correla- tions between biochemical defects and pathological conditions. For instance, *alcaptonuria* is an inherited metabolic disorder caused by the absence of homogentisate oxidase. In 1902, Archibald Garrod showed that alcaptonuria is transmitted as a single recessive Mendelian trait. Furthermore, he recognized that homogentisate is a normal intermediate in the degradation of phenylalanine and tyrosine (see Figure 23.29) and that it accumulates in alcaptonuria because its degradation is blocked. He concluded that "the splitting of the benzene ring in normal metabolism is the work of a special enzyme, that in congenital alcaptonuria this enzyme is wanting." Homogentisate accumulates and is excreted in the urine, which turns dark on standing as homogentisate is oxidized and polymerized to a melanin-like substance.

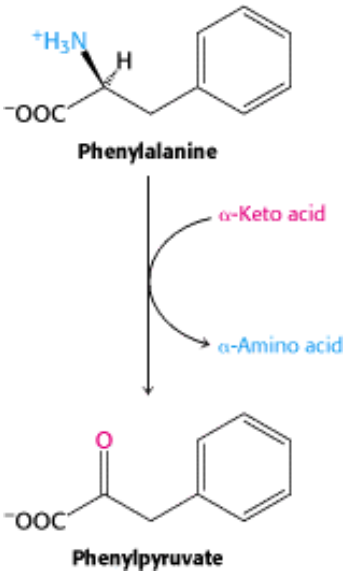


Although alcaptonuria is a relatively harmless condition, such is not the case with other errors in amino acid metabolism. In *maple syrup urine disease*, the oxidative decarboxylation of α-ketoacids derived from valine, isoleucine, and leucine is blocked because the branched-chain dehydrogenase is missing or defective. Hence, the levels of these α-ketoacids and the branched-chain amino acids that give rise to them are markedly elevated in both blood and urine. Indeed, the urine of patients has the odor of maple syrup—hence the name of the disease (also called *branched-chain ketoaciduria*). Maple syrup urine disease usually leads to mental and physical retardation unless the patient is placed on a diet low in valine,

isoleucine, and leucine early in life. The disease can be readily detected in newborns by screening urine samples with 2,4-dinitrophenylhydrazine, which reacts with α-ketoacids to form 2,4-dinitrophenylhydrazone derivatives. A definitive diagnosis can be made by mass spectrometry.



Phenylketonuria is perhaps the best known of the diseases of amino acid metabolism. Phenylketonuria is caused by an *absence or deficiency of phenylalanine hydroxylase* or, more rarely, of its tetrahydrobiopterin cofactor. *Phenylalanine accumulates in all body fluids because it cannot be converted into tyrosine.* Normally, three-quarters of the phenylalanine is converted into tyrosine, and the other quarter becomes incorporated into proteins. Because the major outflow pathway is blocked in phenylketonuria, the blood level of phenylalanine is typically at least 20-fold as high as in normal people. Minor fates of phenylalanine in normal people, such as the formation of phenylpyruvate, become major fates in phenylketonurics.



Indeed, the initial description of phenylketonuria in 1934 was made by observing the reaction of phenylpyruvate with $FeCl_3$, which turns the urine olive green. *Almost all untreated phenylketonurics are severely mentally retarded.* In fact, about 1% of patients in mental institutions have phenylketonuria. The brain weight of these people is below normal, myelination of their nerves is defective, and their reflexes are hyperactive. The life expectancy of untreated phenylketonurics is drastically shortened. Half are dead by age 20 and three-quarters by age 30. *The biochemical basis of their mental retardation is an enigma.*

Phenylketonurics appear normal at birth, but are severely defective by age 1 if untreated. The therapy for phenylketonuria is a *low phenylalanine diet.* The aim is to provide just enough phenylalanine to meet the needs for

growth and replacement. Proteins that have a low content of phenylalanine, such as casein from milk, are hydrolyzed and phenylalanine is removed by adsorption. A low phenylalanine diet must be started very soon after birth to prevent irreversible brain damage. In one study, the average IQ of phenylketonurics treated within a few weeks after birth was 93; a control group treated starting at age 1 had an average IQ of 53.

Early diagnosis of phenylketonuria is essential and has been accomplished by mass screening programs. The phenylalanine level in the blood is the preferred diagnostic criterion because it is more sensitive and reliable than the $FeCl_3$ test. Prenatal diagnosis of phenylketonuria with DNA probes has become feasible because the gene has been cloned and many mutations have been pinpointed to sites in the protein (see Figure 23.31). Interestingly, whereas some mutations affect the activity of the enzyme, others do not affect the activity itself but, instead, decrease the enzyme concentration. These mutations lead to degradation of the enzyme, at least in part by the ubiquitin-proteasome pathway.

The incidence of phenylketonuria is about 1 in 20,000 newborns. The disease is inherited in an *autosomal recessive* manner. Heterozygotes, who make up about 1.5% of a typical population, appear normal. Carriers of the phenylketonuria gene have a reduced level of phenylalanine hydroxylase, as indicated by an increased level of phenylalanine in the blood. However, this criterion is not absolute, because the blood levels of phenylalanine in carriers and normal people overlap to some extent. The measurement of the kinetics of the disappearance of intravenously administered phenylalanine is a more definitive test for the carrier state. It should be noted that a high blood level of phenylalanine in a pregnant woman can result in abnormal development of the fetus. This is a striking example of maternal-fetal relationships at the molecular level. Table 23.3 lists some other diseases of amino acid metabolism.

Table 23.3. Inborn errors of amino acid metabolism

Disease	Enzyme deficiency	Symptoms
Citrullinema	Arginosuccinate lyase	Lethergy, siezures, reduced muscle tension
Tyrosinemia	Various enzymes of tyrosine degradation	Weakness, self-mutilation, liver damage, mental retardation
Albinism	Tyrosinase	Absence of pigmentation
Homocystinuria	Cystathionine β-synthase	Scoliosis, muscle weakness, mental retardation, thin blond hair
Hyperlysinemia	α-Aminoadipic semialdehyde dehydrogenase	Seizures, mental retardation, lack of muscle tone, ataxia

Summary

Proteins Are Degraded to Amino Acids

Dietary protein is digested in the intestine, producing amino acids that are transported throughout the body. Cellular proteins are degraded at widely variable rates, ranging from minutes to the life of the organism.

Protein Turnover Is Tightly Regulated

The turnover of cellular proteins is a regulated process requiring complex enzyme systems. Proteins to be degraded are conjugated with ubi-quitin, a small conserved protein, in a reaction driven by ATP hydrolysis. The ubiquitin conjugating system is composed of three distinct enzymes. A large, barrel-shaped complex called the proteasome digests the

ubiquitinated proteins. The proteasome also requires ATP hydrolysis to function. The resulting amino acids provide a source of precursors for protein, nucleotide bases, and other nitrogenous compounds.

The First Step in Amino Acid Degradation Is the Removal of Nitrogen

Surplus amino acids are used as metabolic fuel. The first step in their degradation is the removal of their α-amino groups by transamination to an α-ketoacid. Pyridoxal phosphate is the coenzyme in all aminotransferases and in many other enzymes catalyzing amino acids transformations. The α-amino group funnels into α-ketoglutarate to form glutamate, which is then oxidatively deaminated by glutamate dehydrogenase to give $NH_4^+$and α-ketoglutarate. NAD^+ or $NADP^+$ is the electron acceptor in this reaction.

Ammonium Ion Is Converted into Urea in Most Terrestrial Vertebrates

The first step in the synthesis of urea is the formation of carbamoyl phosphate, which is synthesized from CO_2, NH_4^+, and two molecules of ATP by carbamoyl phosphate synthetase. Ornithine is then carbamoylated to citrulline by orthinine transcarbamoylase. These two reactions take place in mitochondria. Citrulline leaves the mitochondrion and condenses with aspartate to form argininosuccinate, which is cleaved into arginine and fumarate. The other nitrogen atom of urea comes from aspartate. Urea is formed by the hydrolysis of arginine, which also regenerates ornithine. Some enzymatic deficiencies of the urea cycle can be bypassed by supplementing the diet with arginine or compounds that form conjugates with glycine and glutamine.

Carbon Atoms of Degraded Amino Acids Emerge as Major Metabolic Intermediates

The carbon atoms of degraded amino acids are converted into pyruvate, acetyl CoA, acetoacetate, or an intermediate of the citric acid cycle. Most amino acids are solely glucogenic, two are solely ketogenic, and a few are both ketogenic and glucogenic. Alanine, serine, cysteine, glycine, threonine, and tryptophan are degraded to pyruvate. Asparagine and aspartate are converted into oxaloacetate. α-Ketoglutarate is the point of entry for glutamate and four amino acids (glutamine, histidine, proline, and arginine) that can be converted into glutamate. Succinyl CoA is the point of entry for some of the carbon atoms of three amino acids (methionine, isoleucine, and valine) that are degraded through the intermediate methylmalonyl CoA. Leucine is degraded to acetoacetyl CoA and acetyl CoA. The breakdown of valine and isoleucine is like that of leucine. Their α-ketoacid derivatives are oxidatively decarboxylated by the branched-chain α-ketoacid dehydrogenase.

The rings of aromatic amino acids are degraded by oxygenases. Phenylalanine hydroxylase, a monooxygenase, uses tetrahydrobiopterin as the reductant. One of the oxygen atoms of O_2 emerges in tyrosine and the other in water. Subsequent steps in the degradation of these aromatic amino acids are catalyzed by dioxygenases, which catalyze the insertion of both atoms of O_2 into organic products. Four of the carbon atoms of phenylalanine and tyrosine are converted into fumarate, and four emerge in acetoacetate.

Inborn Errors of Metabolism Can Disrupt Amino Acid Degradation

Errors in amino acid metabolism served as sources of some of the first insights into the correlation between pathology and biochemistry. Although there are many hereditary errors of amino acid metabolism, phenylketonuria is the best known. This condition is the result of the accumulation of high levels of phenylalanine in the body fluids. By unknown mechanisms, this accumulation results in mental retardation unless the afflicted are placed on low phenylalanine diets immediately after birth.

Key Terms

ubiquitin

proteasome

aminotransferase (transaminase)

glutamate dehydrogenase

pyridoxal phosphate (PLP)

pyridoxamine phosphate (PMP)

alanine cycle

urea cycle

carbamoyl phosphate synthetase

N-acetylglutamate

ketogenic amino acid

glucogenic amino acid

biopterin

phenylketonuria

Problems

1. *Wasted energy?* Protein hydrolysis is an exergonic process, yet the 26S proteasome is dependent on ATP hydrolysis for activity.

(a) Although the exact function of the ATPase activity is not known, suggest some likely functions.

(b) Small-peptides can be hydrolyzed without the expenditure of ATP. How does this information concur with your answer to part *a*?

See answer

2. *Keto counterparts.* Name the α-ketoacid that is formed by transamination of each of the following amino acids:

(a) Alanine

(b) Aspartate

(c) Glutamate

(d) Leucine

(e) Phenylalanine

(f) Tyrosine

See answer

3. *A versatile building block.* (a) Write a balanced equation for the conversion of aspartate into glucose through the intermediate oxaloacetate. Which coenzymes participate in this transformation? (b) Write a balanced equation for the conversion of aspartate into oxaloacetate through the intermediate fumarate.

See answer

4. *The benefits of specialization.* The archaeal proteasome contains 14 identical active β subunits, whereas the eukaryotic proteasome has 7 distinct β subunits. What are the potential benefits of having several distinct active subunits?

See answer

5. *Propose a structure.* The 19S subunit of the proteasome contains 6 subunits that are members of the AAA ATPase family. Other members of this large family are associated into homohexamers with six-fold symmetry. Propose a structure for the AAA ATPases within the 19S proteasome. How might you test and refine your prediction?

See answer

6. *Effective electron sinks.* Pyridoxal phosphate stabilizes carbanionic intermediates by serving as an electron sink. Which other prosthetic group catalyzes reactions in this way?

See answer

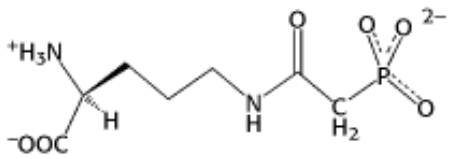
7. *Helping hand.* Propose a role for the positively charged guanidinium nitrogen atom in the cleavage of argininosuccinate into arginine and fumarate.

See answer

8. *Completing the cycle.* Four high-transfer-potential phosphoryl groups are consumed in synthesizing urea according to the stoichiometry given in Section 23.4.2. In this reaction, aspartate is converted into fumarate. Suppose that fumarate is converted back into aspartate. What is the resulting stoichiometry of urea synthesis? How many high-transfer-potential phosphoryl groups are spent?

See answer

9. *Inhibitor design.* Compound A has been synthesized as a potential inhibitor of a urea-cycle enzyme. Which enzyme do you think compound A might inhibit?



Compound A

See answer

10. *Ammonia toxicity.* Glutamate is an important neurotransmitter whose levels must be carefully regulated in the brain. Explain how a high concentration of ammonia might disrupt this regulation. How might a high concentration of ammonia alter the citric acid cycle?

See answer

11. *A precise diagnosis.* The urine of an infant gives a positive reaction with 2,4-dinitrophenylhydrazine. Mass spectrometry shows abnormally high blood levels of pyruvate, α-ketoglutarate, and the α-ketoacids of valine, isoleucine, and leucine. Identify a likely molecular defect and propose a definitive test of your diagnosis.

See answer

12. *Therapeutic design.* How would you treat an infant who is deficient in argininosuccinate synthetase? Which molecules would carry nitrogen out of the body?

See answer

13. *Sweet hazard.* Why should phenylketonurics avoid using aspartame, an artificial sweetener? (Hint: Aspartame is l-aspartyl-l-phenylalanine methyl ester.)

See answer

14. *Déjà vu. N*-Acetylglutamate is required as a cofactor in the synthesis of carbamoyl phosphate. How might *N*-acetylglutamate be synthesized from glutamate?

See answer

Mechanism Problems

15. *Serine dehydratase.* Write out a complete mechanism for the conversion of serine into aminoacrylate catalyzed by serine dehydratase.

See answer

16. *Serine racemase.* The nervous system contains a substantial amount of d-serine, which is generated from l-serine by serine racemase, a PLP-dependent enzyme. Propose a mechanism for this reaction. What is the equilibrium constant for the reaction l-serine ⇌ d-serine?

See answer

Chapter Integration Problems

17. *Double duty.* Degradation signals are commonly located in protein regions that also facilitate protein-protein interactions. Explain why this coexistence of two functions in the same domain might be useful.

See answer

18. *Fuel choice.* Within a few days after a fast begins, nitrogen excretion accelerates to a higher-than-normal level. After a few weeks, the rate of nitrogen excretion falls to a lower level and continues at this low rate. However, after the fat stores have been depleted, nitrogen excretion rises to a high level.

(a) What events trigger the initial surge of nitrogen excretion?

(b) Why does nitrogen excretion fall after several weeks of fasting?

(c) Explain the increase in nitrogen excretion when the lipid stores have been depleted.

See answer

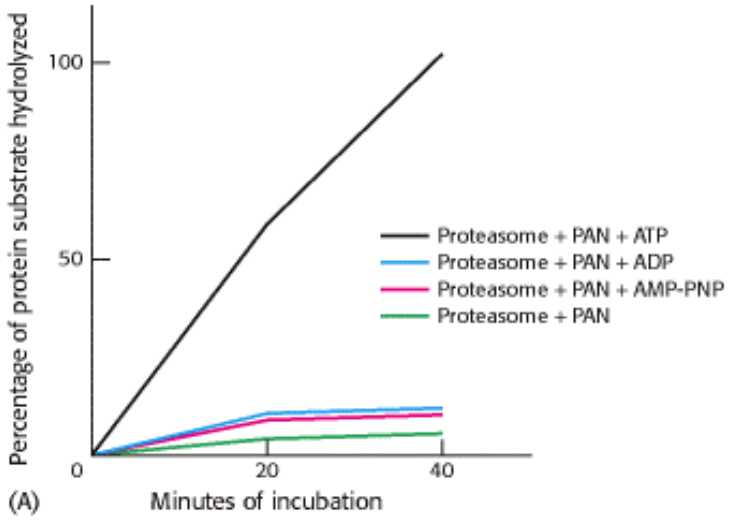
19. *Isoleucine degradation.* Isoleucine is degraded to acetyl CoA and succinyl CoA. Suggest a plausible reaction sequence, based on reactions discussed in the text, for this degradation pathway.

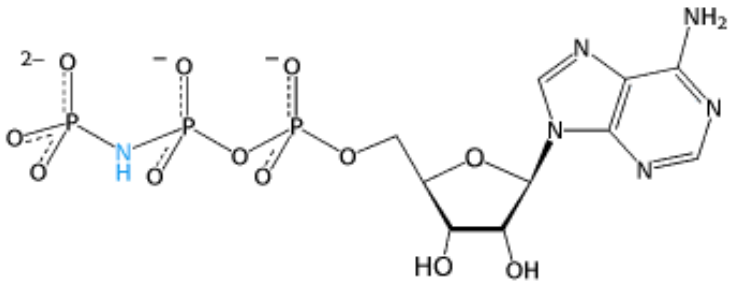
See answer

Data Interpretation Problem

20. *Another helping hand.* In eukaryotes, the 20S proteasome in conjunction with the 19S component degrades ubiquitinated proteins with the hydrolysis of a molecule of ATP. Archaea lack ubiquitin and the 26S proteasome but do contain a 20S proteasome. Some archaea also contain an ATPase that is homologous to the ATPases of the eukaryotic 19S component. This archaeal ATPase activity was isolated as a 650-kd complex (called PAN) from the archaeon *Thermoplasma*, and experiments were performed to determine if PAN could enhance the activity of the 20S proteasome from *Thermoplasma* as well as other 20S proteasomes.

Protein degradation was measured as a function of time and in the presence of various combinations of components. Graph A shows the results.





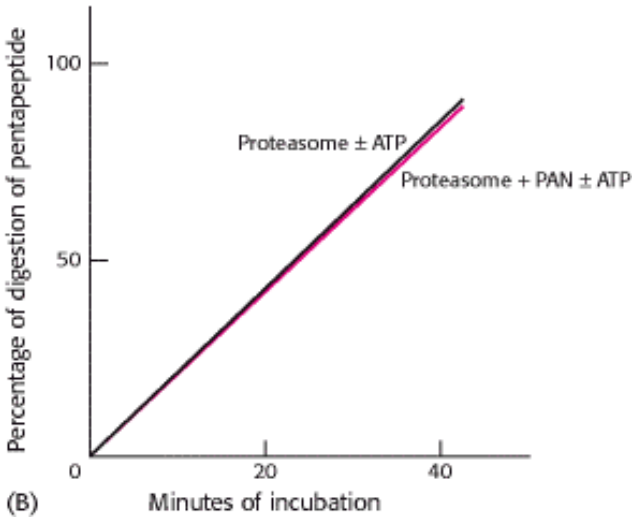
AMP-PNP

(a) What is the effect of PAN on archaeal proteasome activity in the absence of nucleotides?

(b) What is the nucleotide requirement for protein digestion?

(c) What evidence suggests that ATP hydrolysis, and not just the presence of ATP, is required for digestion?

A similar experiment was performed with a small peptide as a substrate for the proteasome instead of a protein. The results obtained are shown in graph B.



(B)

(d) How do the requirements for peptide digestion differ from those of protein digestion?

(e) Suggest some reasons for the difference.

The ability of PAN from the archaeon *Thermoplasma* to support protein degradation by the 20S proteasomes from the archeaon *Methanosarcina* and rabbit muscle was then examined.

Additions	Percentage of digestion of protein substrate (20S proteasome source)		
	Thermoplasma	*Methanosarcina*	Rabbit-muscle
None	11	10	10
PAN	8	8	8
PAN + ATP	100	40	30
PAN + ADP	12	9	10

(f) Can the *Thermoplasma* PAN augment protein digestion by the proteasomes from other organisms?

(g) What is the significance of the stimulation of rabbit muscle proteasome by *Thermoplasma* PAN?

[Data from P. Zwickl, D. Ng, K. M. Woo, H.-P. Klenk, and A. L. Goldberg. An archaebacterial ATPase, homologous to ATPase in the eukaryotic 26S proteasome, activates protein breakdown by 20S proteasomes. *J. Biol. Chem.* 274(1999): 26008–26014.]

See answer

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III. Synthesizing the Molecules of Life