Chapter 3—
Proteins II:
Structure—Function Relationships in Protein Families

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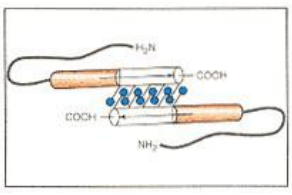


3.1— Overview

In Chapter 2 we discussed the fundamentals of protein architecture, including structural organization and physical properties of the amino acid constituents, hierarchical organization of primary, secondary, supersecondary, tertiary, and quaternary structure, and energetic forces that hold these molecules together and provide the flexibility observed in their dynamic motion. Computational and experimental tools were introduced that enable the analysis of high-resolution structural features and their conformational response to perturbations, which may be a simple alteration of the solution environment or aspects of their interactions with other molecules that define their biological function. The concept that structure and function are interrelated was introduced through examples of conservation of primary structure with function, and the reoccurrence of elements of secondary, supersecondary, tertiary, and quaternary structural patterns in molecules that may not share similar functional or evolutionary origin.

In this chapter we examine the specific relationships between structure and function in four **protein families**: immunoglobulins, serine proteases, DNA-binding proteins, and hemoglobins. We pursue this study through the examination of the variability in amino acid sequence, structural organization, and biological function. The significance of the structure–function relationship can best be appreciated through observation of the range of such variations within specific protein families.

The **immunoglobulin family** provides examples of multidomain architecture that supports recognition and binding to foreign molecules and leads to their sequestration. Diversity among family members is the source of specific molecular recognition and individual binding capabilities.

The **serine proteases** provide examples of a family of enzymes that appear to have diverged to perform unique physiological functions, frequently highly organized within enzyme cascade processes. Their inherent similarities in catalytic mechanism and three-dimensional structure are a common link.

DNA-binding proteins are multifamilies of proteins that bind to regulatory sites in DNA and regulate gene expression, an amazing feat as the mammalian genome contains approximately 100,000 unique genes. These proteins contain unusual supersecondary structure motifs that allow them to selectively bind regulatory sites of specific genes.

The **hemoglobin** family offers examples of a highly fine-tuned system that can accommodate small substitutions or mutations, many of which have been studied as to their clinical implications. This family reveals the potential diversity of amino acid sequence substitutions that can be tolerated and allow the protein to function in an acceptable physiological manner.

3.2— Antibody Molecules: The Immunoglobulin Superfamily

Antibody molecules are produced in response to invasion by foreign compounds that can be proteins, carbohydrates, and nucleic acid polymers. An antibody molecule noncovalently associates with the foreign substance, initiating a process by which the foreign substance can be eliminated from the organism.

Molecules that induce antibody production are **antigens** and may contain multiple antigenic determinants, small regions of the antigen molecule that elicit the production of a specific antibody to which the antigen binds. In proteins, an antigenic determinant may comprise only six or seven amino acids.

A **hapten** is a small molecule that cannot alone elicit production of specific antibodies but when covalently attached to a larger molecule it acts as an antigenic determinant and induces antibody synthesis. Whereas hapten molecules need attachment to a larger molecule to elicit antibody synthesis, when

detached from their carrier, they will retain the ability to bind strongly to antibody.

It is estimated that each human can potentially produce about 1×10^8 different antibody structures. All antibodies, however, have a similar structure. The determination of the structure has been accomplished from studies of immunoglobulin primary structures and X-ray diffraction that show the three-dimensional structure of the antibody molecule alone and in complex with antigen.

Structural studies of proteins require pure homogeneous preparations. Such samples of antibodies are extremely difficult to isolate from blood because of the wide diversity of antibody molecules present. Homogeneous antibodies can be obtained, however, by the monoclonal hybridoma technique in which mouse myeloma cells are fused with mouse antibody-producing B lymphocytes to construct immortalized hybridoma cells that express a single antibody.

Antibody (Immunoglobulin) Molecules Consist of Four Polypeptide Chains

Antibody molecules are glycoproteins with four polypeptide chains, two identical copies of each of two nonidentical polypeptide chains. Two light chains (L) of identical sequence combine with two heavy chains (H) of identical sequence to form the structure $(LH)_2$. In the most common immunoglobulin type, IgG, the H chains have approximately 440 amino acids (50 kDa). The smaller L polypeptide chains contain about 220 amino acids (25 kDa). The four chains are covalently interconnected by disulfide bonds (Figures 3.1 and 3.2). Each H chain is associated with an L chain such that the NH_2-terminal ends of both chains are near each other. Since the L chain is half the size of the H chain, only the NH_2-terminal half of the H chain is associated with the L chain.

In the other classes of immunoglobulins (Table 3.1) the H chains are slightly longer than those of the IgG class. A variable amount of carbohydrate (2–12%, depending on immunoglobulin class) is attached to the H chain.

Constant and Variable Regions of Primary Structure

Comparison of amino acid sequences of antibody molecules elicited by different antigens shows regions of sequence homology and other regions of sequence variability. In particular, sequences of the NH_2-terminal half of L chains and the

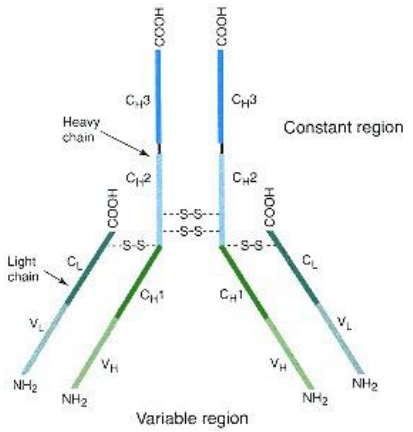


Figure 3.1
Linear representation of four-chain IgG antibody molecule.
Two H chains and two L chains are co-oriented in their COOH-terminal to NH_2-terminal directions, as shown. Interchain disulfide bonds link heavy (H) chains, and light (L) chains to the H chains. Domains of the constant (C) region of the H chain are C_H1, C_H2, and C_H3. The constant region of the L chain is designated C_L, and variable (V) regions are V_H and V_L of H and L chains, respectively. Based on figure by Burton, D. R. In: F. Calabi and M. S. Neuberger (Eds.), *Molecular Genetics of Immunoglobulin*. Amsterdam: Elsevier, 1987, pp. 1–50.

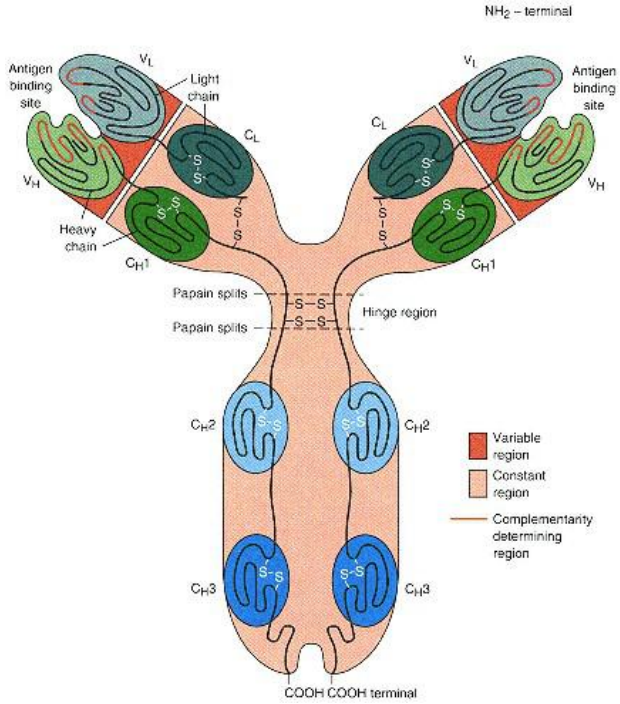


Figure 3.2
Diagrammatic structure for IgG.
Light chains (L) are divided into domains V_L (variable amino acid sequence) and C_L (constant amino acid sequence). Heavy chains (H) are divided into domains V_H (variable amino acid sequence) and C_H1, C_H2, and C_H3. Antigen-binding sites are V_H–V_L. "Hinge" polypeptides interconnect domains.
Positions of inter- and intrachain cystine bonds are shown.
From Cantor, C. R. and Schimmel, P. R. *Biophysical Chemistry,* Part I. San Francisco: Freeman, 1980. Re-printed with permission of Mr. Irving Geis, New York.

NH_2-terminal quarter of H chains are highly variable between different antibody molecules. These NH_2-terminal segments are the **variable (V) regions** and designated V_H and V_L domains of H and L chains, respectively. Within these V domains certain segments are "hypervariable." Three **hypervariable regions** of between 5 and 7 residues in the V_L domain and three or four hypervariable regions of between 6 and 17 residues in the V_H domain are commonly found. The hypervariable sequences are also termed the **complementarity-determining regions (CDRs)** as they form the antigen-binding site complementary to the topology of the antigen structure.

In contrast, the COOH-terminal three-quarters of H chains and the COOH-terminal half of L chains are homologous in sequence with other H or L chains

TABLE 3.1 Immunoglobulin Classes

Classes of Immunoglobulin	*Approximate Molecular Mass*	*H Chain Isotype*	*Carbohydrate by Weight (%)*	*Concentration in Serum (mg 100 mL^{-1})*
IgG	150,000	γ, 53,000	2–3	600–1800
IgA	170,000–720,000[a]	α, 64,000	7–12	90–420
IgD	160,000	δ, 58,000		0.3–40
IgE	190,000	ε, 75,000	10–12	0.01–0.10
IgM	950,000[a]	μ, 70,000	10–12	50–190

[a] Forms polymer structures of basic structural unit.

of the same class. These **constant (C) regions** with a homologous primary structure are designated C_H and C_L in the H and L chains, respectively.

The C_H regions determine the antibody class, provide for binding of complement proteins (see Clin. Corr. 3.1), and are the site necessary for antibodies to cross the placental membrane. The V regions determine the antigen specificity of the antibody molecule.

Immunoglobulins in a Single Class Contain Common Homologous Regions

Differences in sequence of the C_H regions between immunoglobulin classes are responsible for the characteristics of each class. In some cases, the C_H sequence promotes the polymerization of antibody molecules of the basic molecular structure $(LH)_2$. Thus antibodies of the IgA class are often covalently linked dimeric structures $[(LH)_2]_2$. Similarly, IgM molecules are pentamers $[(LH)_2]_5$. The different H chains, designated τ, α, δ, ε and μ, occur in IgG, IgA, IgM, IgD, and IgE classes, respectively (Table 3.1; see Clin. Corr. 3.2). Two types of L chain sequences are synthesized, designated lambda (λ) and kappa (κ) chains, either of which are found combined with the five classes of H chains.

IgG is the major immunoglobulin in plasma. Biosynthesis of a specific IgG in significant concentrations takes about 10 days after exposure to a new antigen (see Clin. Corr. 3.3). In the absence of an initially high concentration of IgG to a specific antigen, antibodies of the IgM class, which are synthesized at faster

CLINICAL CORRELATION 3.1

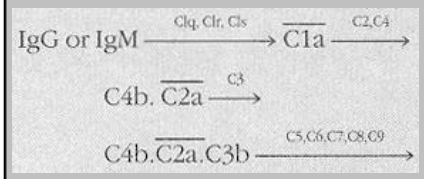
The Complement Proteins

At least 11 distinct complement proteins exist in plasma. They are activated by IgG or IgM antibody binding to antigens on the outer cell membrane of invading bacterial cells, protozoa, or tumor cells. After the immunoglobulin-binding event, the 11 complement proteins are sequentially activated and associate with the cell membrane to cause a lysis of the membrane and death of the target cell.

Many complement proteins are precursors of proteolytic enzymes that are present in a nonactive form prior to activation. Upon their activation, they will, in turn, activate a succeeding protein of the pathway by the hydrolysis of a specific peptide bond in the second protein, leading to a cascade phenomenon. Activation of enzymes by specific proteolysis (i.e., hydrolysis of a specific peptide bond) is an important general method for activating extracellular enzymes. For example, the enzymes that catalyze blood clot formation, induce fibrinolysis of blood clots, and digest dietary proteins in the gut are all activated by a specific proteolysis catalyzed by a second enzyme (see pp. 964, 1071).

Upon association to a cellular antigen the exposure of a complement-binding site in the antibody's Fc region occurs and causes the binding of the C1 complement proteins, which are a protein complex composed of three individual proteins: Clq, Clr, and Cls. Clr and Cls undergo a conformational change and become active enzymes on the cell surface. The activated Cl complex (Cla) hydrolyzes a peptide bond in complement proteins C2 and C4, which then also associate on the cell surface. The now active C2–C4 complex has a proteolytic activity that hydrolyzes a peptide bond in complement protein C3. Activated C3 protein binds to the cell surface and the activated C2–C4–C3 complex activates protein C5. Activated protein C_5 will associate with complement proteins C6, C7, C8, and six molecules of complement protein C9. This multiprotein complex binds to the cell surface and initiates membrane lysis.

The mechanism is a cascade in which amplification of the trigger event occurs. In summary, activated C1 can activate a number of molecules of C4–C2–C3, and each activated C4–C2–C3 complex can, in turn, activate many molecules of C5 to C9. The reactions of the classical complement pathway are summarized below, where "a" and "b" designate the proteolytically modified proteins and a line above a protein indicates an enzyme activity.



There is an "alternative pathway" for C3 complement activation, initiated by aggregates of IgA or by bacterial polysaccharide in the absence of immunoglobulin binding to cell membrane antigens. This pathway involves the proteins properdin, C3 proactivator convertase, and C3 proactivator.

A major role of the complement systems is to generate opsonins—an old term for proteins that stimulate phagocytosis by neutrophils and macrophages. The major opsonin is C3b; macrophages have specific receptors for this protein. Patients with inherited deficiency of C3 are subject to repeated bacterial infections.

Colten, H. R., and Rosen, F. S. Complement deficiencies. *Annu. Rev. Immunol.* 10:809, 1992; and Morgan, B. P. Physiology and pathophysiology of complement: progress and trends. *Crit. Rev. Clin. Lab. Sci.* 32:265, 1995.

CLINICAL CORRELATION 3.2

Functions of Different Antibody Classes

The IgA class of immunoglobulins is found primarily in the mucosal secretions (bronchial, nasal, and intestinal mucous secretions, tears, milk, and colostrum). These immunoglobulins are the initial defense against invading viral and bacterial pathogens prior to their entry into plasma or other internal space.

The IgM class is found primarily in plasma. They are the first antibodies elicited in significant quantity on the introduction of a foreign antigen into a host's plasma. IgM antibodies promote phagocytosis of microorganisms by macrophage and polymorphonuclear leukocytes and are also potent activators of complement (see Clin. Corr. 3.1). IgM antibodies occur in many external secretions but at levels lower than those of IgA.

The IgG class occurs in high concentration in plasma. Their response to foreign antigens takes a longer period of time than that of IgM. At maximum concentration they are present in significantly higher concentration than IgM. Like IgM, IgG antibodies promote phagocytosis in plasma and activate complement.

The normal biological functions of the IgD and IgE classes of immunoglobulins are not known; however, the IgE antibodies play an important role in allergic responses such as anaphylactic shock, hay fever, and asthma.

Immunoglobulin deficiency usually causes increased susceptibility to infection. X-linked agammaglobulinemia and common variable immunodeficiency are two examples. The commonest disorder is selective IgA deficiency, which results in recurrent infections of sinuses and the respiratory tract.

Rosen, F. S., Cooper, M. D., and Wedgewood, R. J. P. The primary immunodeficiencies. *N. Engl. J. Med.* 311:235 (Part 1); 300 (Part II), 1984.

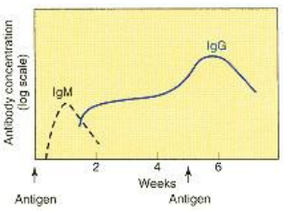


Figure 3.3
Time course of specific antibody IgM and IgG response to added antigen.
Based on a figure in Stryer, L. *Biochemistry*. San Francisco: Freeman, 1988, p. 890.

rates, will associate with the antigen and serve as the first line of defense until large quantities of IgG are produced (Figure 3.3; see Clin. Corr. 3.3).

Repeating Amino Acid Sequences and Homologous Three-Dimensional Domains Occur within an Antibody

Within each of the polypeptide chains of an antibody molecule is a repeating pattern of amino acid sequences. For the IgG class, the repetitive pattern is observed between segments of approximately 110 amino acids within both L and H chains. This homology is far from exact, but clearly a number of amino acids match identically following alignment of 110 amino acid segments. Other amino acids are matched in the sequence by having similar nonpolar or polar side chains. As the H chains are about 440 amino acids in length, the repetition of the homologous sequence occurs four times along an immunoglobulin H chain. Based on this sequence pattern, the chain is divided into one V_H region and three C_H regions (designated C_H1, C_H2, and C_H3) (see Figures 3.1 and 3.2). The L chain of about 220 amino acids is divided into one V_L region and one

CLINICAL CORRELATION 3.3

Immunization

An immunizing vaccine can consist of killed bacterial cells, inactivated viruses, killed parasites, a nonvirulent form of live bacterium related to a virulent bacterium, a denatured bacterial toxin or recombinant protein. The introduction of a vaccine into a human can lead to protection against virulent forms of microorganisms or toxic agents that contain the same antigen. Antigens in nonvirulent material not only cause the differentiation of lymphoid cells so that they produce antibody toward the foreign antigen but also cause differentiation of some lymphoid cells into memory cells. Memory cells do not secrete antibody but place antibodies to the antigen onto their outer surface, where they act as future sensors for the antigen. These memory cells are like a longstanding radar for the potentially virulent antigen. On reintroduction of the antigen at a later time, the binding of the antigen to the cell surface antibody in the memory cells stimulates the memory cell to divide into antibody-producing cells as well as new memory cells. This reduces the time for antibody production that is required on introduction of an antigen and increases the concentration of antigen-specific antibody produced. It is the basis for the protection provided by immunization.

Recently introduced vaccines for adults include pneumococcal vaccine (to prevent pneumonia due to *Diplococcus pneumoniae*), hepatitis B vaccine, and influenza vaccine. The latter changes each year to account for antigenic variation in the influenza virus.

Flexner, C. New approaches to vaccination. *Adv. Pharmacol.* 21:51, 1990; and Sparling, P. F., Elkins, C., Wyrick, P. B., and Cohen, M. S. Vaccines for bacterial sexually transmitted infections: a realistic goal? *Proc. Natl. Acad. Sci. U.S.A.* 91:2456, 1994.

C_L region. Each of these sequence repeats contains an intrachain disulfide bond linking two cysteines (Figure 3.2).

Each of the 110 amino acid segments form separate structural domains of similar tertiary structure as shown by X-ray diffraction studies. Each 110 segment of the H and L chains folds into a supersecondary structure with a unique but similar arrangement of antiparallel β-strands, which generates a motif known as an **immunoglobulin fold** (Figure 3.4). This motif consists of 7 to 9 polypeptide strands that form two antiparallel β-sheets that are aligned face-to-face. Globular domains result from the strong interaction between two immunoglobulin folds on two separate chains (Figure 3.5). The associations are between domains V_L–V_H and C_L–C_H1 in the H and L chains. In the C-terminal half of the H chains, the two chains associate to generate domains C_H2–C_H2 and C_H3–C_H3 (Figure 3.2). A "hinge" polypeptide sequence interconnects the two C_L–C_H1 domains with the C_H2–C_H2 domain in the antibody structure. Thus the antibody structure

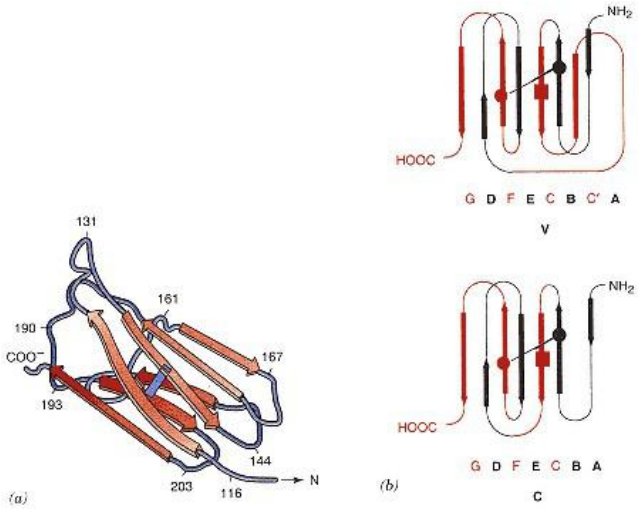


Figure 3.4
Immunoglobulin fold.
(a) Schematic diagram of folding of a C_L domain, showing
-pleated sheet structure. Arrows show strands of -sheet and bar (blue) shows position of cystine bond. Light arrows are for -strands in plane above and dark arrows are -strands in plane below.
(b) Diagrammatic outline of arrangement of
-strands in immunoglobulin fold motif. Examples are for IgG variable and constant regions. Thick arrows indicate -strands and thin lines loops that interconnect the -strands. Circles indicate cysteines that form intradomain disulfide bond. Squares show positions of tryptophan residues that are an invariant component of the core of the immunoglobulin fold. Boldface black letters indicate strands that form one plane of the sheet, while other letters form the parallel plane behind the first plane.
(a) From Edmundson, A. B., Ely, K. R., Abola, E. E., Schiffer, M., and Pavagiotopoulos, N. Biochemistry 14:3953, 1975.

(b) Based on a figure by Calabi, F. In: F. Calabi and M. S. Neuberger (Eds.), *Molecular Genetics of Immunoglobulin*. Amsterdam: Elsevier, 1987, pp. 203–239.

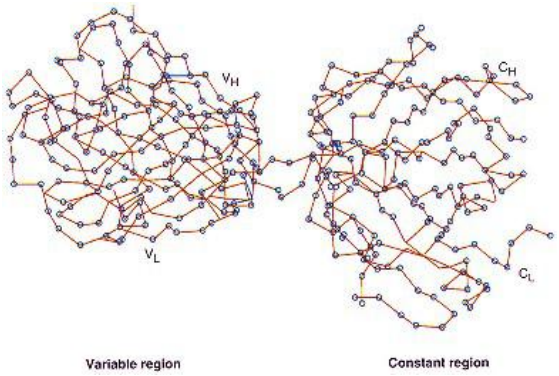


Figure 3.5
α-Carbon (○) structure of Fab fragment of IgG KOL showing V_L–V_H and C_L–C_H1 domains interconnected by the hinge polypeptides.
From Huber, R., Deisenhofer, J., Coleman, P. M., Matsushima, M., and Palm, W. In *The Immune System,* 27th Mosbach Colloquium. Berlin: Springer-Verlag, 1976, p. 26.

exhibits six domains, each domain due to the association of two immunoglobulin folds (Figures 3.2 and 3.6). The NH_2-terminal V_L–V_H domains contain a shallow crevice in the center of a hydrophobic core that binds the antigen. Hypervariable sequences in the V domain crevices form loops that come close together and are the complementarity binding site for the antigen (see Figures 3.6 and 3.7). The sequences of the hypervariable loops give a unique three-dimensional conformation for each antibody that makes it specific to its antigenic determinant. Small changes in conformation of the CDRs occur on antigen binding to V_L–V_H domains, indicating that antigen binding induces an optimum complementary fit to the variable CDR site. Antigen binding may also induce conformational changes between V_L–V_H domains and the other domains that activate effector sites, such as for complement binding to the C_H2–C_H2 domain. The strength of association between antibody and antigen is due to noncovalent forces (see Chapter 2). Complementarity of the structures of the antigenic determinant and antigen-binding site results in extremely high equilibrium affinity constants, between 10^5 and 10^{10} M^{-1} (strength of 7–14 kcal mol^{-1}) for this noncovalent association.

There Are Two Antigen-Binding Sites Per Antibody Molecule

The NH_2-terminal variable (V) domains of each pair of L and H chains (V_L–V_H) comprise an antigen-binding site; thus there are two antigen-binding sites per antibody molecule. The existence of an antigen-binding site in each LH pair is demonstrated by treating antibody molecules with the proteolytic enzyme papain, which hydrolyzes a peptide bond in the hinge peptide of each H chain (see Figures 3.2 and 3.8). The antibody molecule is cleaved into three products. Two are identical and consist of the NH_2-terminal half of the H chain (V_H–C_H1) associated with the full L chain (Figure 3.8). Each of these fragments binds antigen with a similar affinity to that of the intact antibody molecule and is designated an **Fab** (antigen binding) **fragment.** The other product from the papain hydrolysis is the COOH-terminal half of the H chains (C_H2–C_H3) joined together in a single covalent fragment by cystine bonds. This is the **Fc** (crystallizable) **fragment,** which exhibits no binding affinity for the antigen. The L chain can be dissociated from its H chain segment within the Fab fragment by oxidation of disulfide bonds, which eliminates antigen binding. Accordingly, each antigen-binding site must be formed from components of both the L chain (V_L) and the H chain (V_H) domains acting together.

In summary, the major features of antibody structure and antibody–antigen interactions include the following: (1) The polypeptide chains fold into multiple

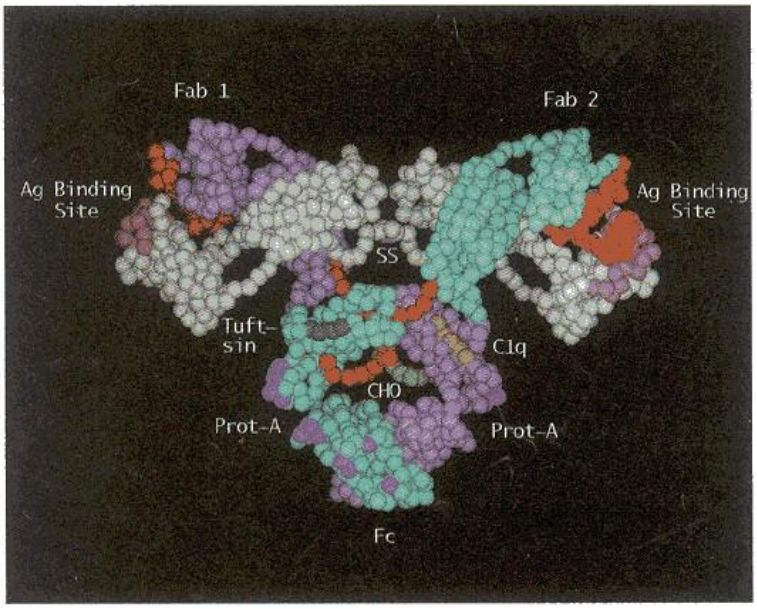


Figure 3.6
Model of an IgG antibody molecule.
Only the -carbons of the structure appear. The two L chains are represented by light gray spheres and the H chains by lavender spheres. Carbohydrates attached to the two C_H2 domains are green and orange. The CDR regions of the V_H–V_L domains are dark red in the H chains and pink in the L chains. The interchain disulfide bond between the L and H chains is a magenta ball-and-stick representation (partially hidden). The heptapeptide hinge between C_H1 and C_H2 domains, connecting the Fab and Fc units, are dark red. The center of the C1q complement site in the C_H2 domains is yellow-green the protein A docking sites at the junction of C_H2 and C_H3 are magenta, and the tuftsin binding site in C_H2 is gray. Tuftsin is a natural tetrapeptide that induces phagocytosis by macrophages and may be transported bound to an immunoglobulin. Protein A is a bacterial protein with a high affinity to immunoglobulins.
Photograph generously supplied by Dr. Allen B. Edmundson, from Guddat, L. W., Shan, L., Fan Z-C., et al. *FASEB J.* 9:101, 1995.

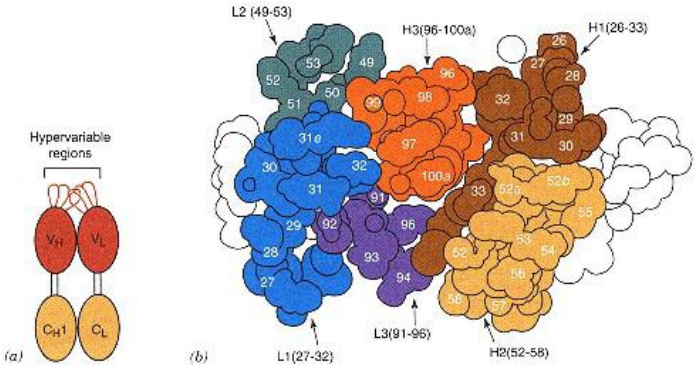


Figure 3.7
Hypervariable loops in immunoglobin.
(a) Schematic diagram showing hypervariable loops (CDRs) in V_L–V_H domain that form the antigen-binding site.
(b) A cut through an antigen-binding site showing contributions of different CDRs using CPK space-filling models of the atoms.
(a)From Branden, C. and Tooze, J. *Introduction to Protein Structure*. New York Garland Publishing, 1991, *p. 187*.
(b) From Branden, C., and Tooze, J. *Introduction to Protein Structure*. New York Garland Publishing, 1991, p. 189, and attributed to Chothia, C. and Lesk, *A. J. Mol. Biol*. 196:914, 1987.

domains, each domain having an immunoglobulin fold supersecondary structure motif. (2) Two immunoglobulin folds on separate chains associate to form the six domains of the basic immunoglobulin structure. The V_L and V_H associate to form the two NH_2-terminal domains that bind to antigen. (3) The antigen-binding site of the V_L–V_H domains is generated by hypervariable loops (CDRs), which form a continuous surface with a complementary topology to the antigenic determinant. (4) The strong interactions between antigen and antibody CDRs are noncovalent and include van der Waals, hydrogen bonding, and hydrophobic interactions. Ionic salt bridges participate in antigen–antibody associations to a much lesser extent. (5) Small conformational changes occur in the V_L–V_H domain upon association of antigen, indicating an "induced-fit" mechanism in association of antigen to antibody. (6) The binding of antigen to the V_L–V_H domains induces conformational changes between binding and distant domains of the antibody. These allosteric movements alter the binding affinity of effector sites in the constant domains such as that for binding of complement protein C1q to the C_H2–C_H2 domain (see Clin. Corr. 3.1).

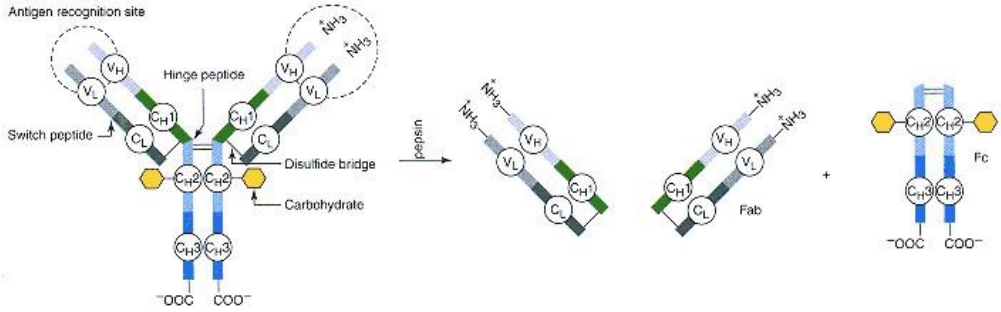


Figure 3.8
Hydrolysis of IgG into two Fab and one Fc fragments by papain, a proteolytic enzyme.

The Genetics of the Immunoglobulin Molecule Have Been Determined

Genes that code for amino acid sequences of human IgG L chains are located on different chromosomes than those for IgG H. The V and C regions of the L and H chains are specified by distinct genes. There are four unique genes that code for the C domains of the H chain in the IgG antibody class. Each gene codes for a complete constant region, thus coding for all the amino acids of the H chain except for the V_H region sequence. These four genes are known as gamma (γ) genes—that is, γ_1, γ_2, γ_3, and γ_4—that give rise to **IgG isotypes** IgG_1, IgG_2, IgG_3, and IgG_4. Figure 3.9 presents the amino acid sequences of three γ-gene proteins. There is a 95% homology in amino acid sequence among the genes.

It is likely that a primordial gene coded for a single segment of approximately 110 amino acids, and **gene duplication** events resulted in the three repeating units within the same gene. Mutations modified the individual sequences so that an exact correspondence in sequence no longer exists. Each

Figure 3.9
Amino acid sequence of the heavy chain constant regions of the IgG heavy chain γ_1, γ_2, and γ_4 genes.
Domains of constant domain C_H1, hinge region H, constant domain C_H2, and constant domain C_H3 are presented. Sequence for $_1$ is fully given and differences in $_2$ and $_4$ from $_1$ sequence are shown using single-letter amino acid abbreviations. Dashed line (–) indicates absence of an amino acid in position correlated with $_1$, in order to better align sequences to show maximum homology. Sequence of $_1$ chain from Ellison, J. W., Berson, B. J., and Hood, L. E. *Nucleic Acid Res.* 10:4071, 1982; and sequences of the $_2$ and $_4$ genes from Ellison, J. and Hood, L. *Proc. Natl. Acad. Sci. U.S.A.* 79:1984, 1982.

immunoglobulin domain has a similar domain length and immunoglobulin folding pattern stabilized by a cystine linkage. Later in evolution gene duplications led to the multiple genes (γ_1, γ_2, γ_3, and γ_4) that code for the constant regions of the IgG class H chains.

The Immunoglobulin Fold Is a Tertiary Structure Found in a Large Family of Proteins with Different Functional Roles

The immunoglobulin fold motif is present in many nonimmunological proteins, which exhibit widely different functions. Based on their structural homology they are grouped into a **protein superfamily** (Figure 3.10). For example, the Class 1 major histocompatibility complex proteins are in this superfamily, they have immunoglobulin fold motif structures consisting of two stacked antiparallel β-sheets enclosing an internal space filled mainly by hydrophobic amino acids. Two cysteines in the structure form a disulfide bond linking the facing β-sheets. Transcription factors NF-κB and p53 also contain an immunoglobulin fold motif. It can be speculated that gene duplication during evolution led to distribution of the structural motif in the functionally diverse protein superfamily.

3.3— Proteins with a Common Catalytic Mechanism: Serine Proteases

Serine proteases are a family of enzymes that utilize a single uniquely activated serine residue in their substrate-binding site to catalytically hydrolyze peptide bonds. This serine can be characterized by the irreversible reaction of its side chain hydroxyl group with diisopropylfluorophosphate (DFP) (Figure 3.11). Of

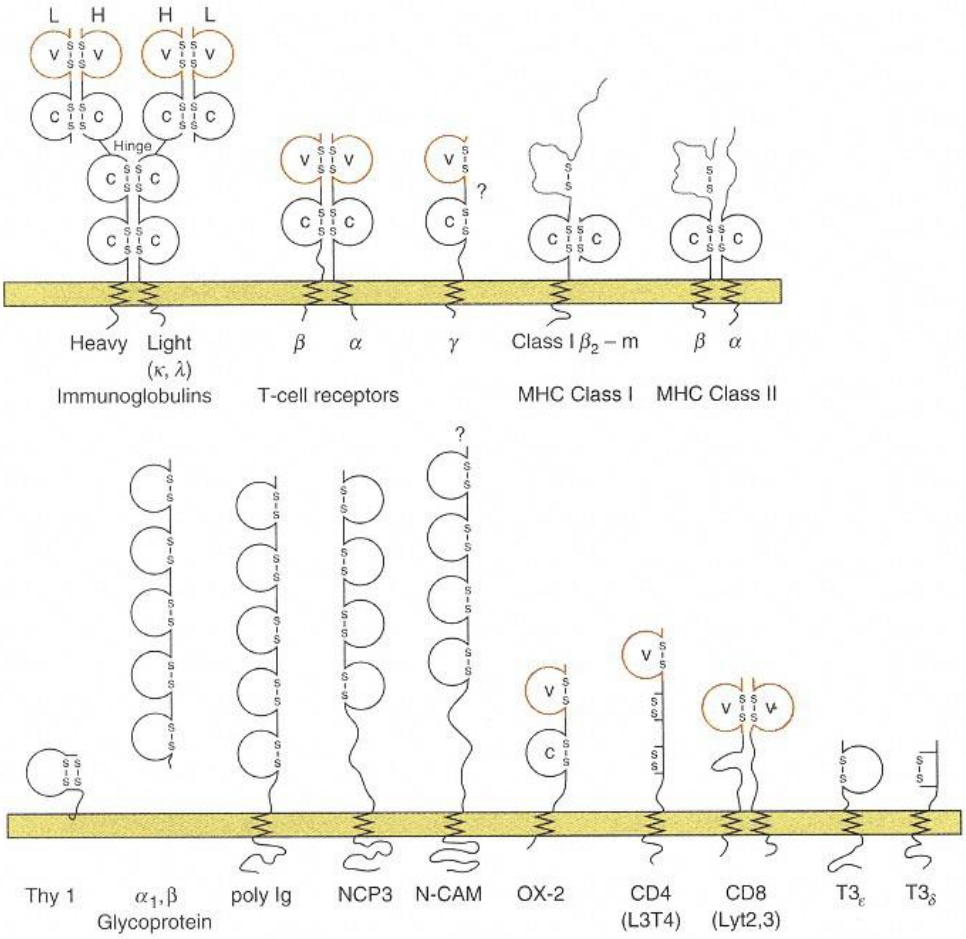


Figure 3.10
Diagrammatic representation of immunoglobulin domain structures from different proteins of immunoglobulin gene superfamily.
Proteins presented include heavy and light chains of immunoglobulins, T-cell receptors, major histocompatibility complex (MHC) Class I and Class II proteins, T-cell accessory proteins involved in Class I (CDS) and Class II (CD4) MHC recognition and possible ion channel formation, a receptor responsible for transporting certain classes of immunoglobulin across mucosal membranes (poly-Ig), $_2$-micro-globulin, which associates with class I molecules, a human plasma protein with unknown function ($_1$/ -glycoprotein), two molecules of unknown function with a tissue distribution that includes lymphocytes and neurons (Thy-1, OX-2), and two brain-specific molecules, neuronal cell adhesion molecule (N-CAM) and neurocytoplasmic protein 3 (NCP3). Reprinted with permission from Hunkapiller, T., and Hood, L. *Nature* 323:15, 1986.

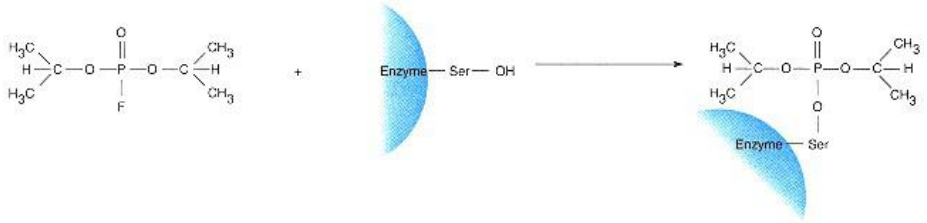


Figure 3.11
Reaction of diisopropylfluorophosphate (DFP) with the active-site serine in a serine protease.

all the serines in the protein, DFP reacts only with the catalytically active serine to form a phosphate ester.

Proteolytic Enzymes Are Classified Based on Their Catalytic Mechanism

Proteolytic enzymes are classified according to their catalytic mechanism. Besides serine proteases, other classes utilize cysteine **(cysteine proteases),** aspartate **(aspartate proteases),** or metal ions **(metallo proteases)** to perform their catalytic function. Proteases that hydrolyze peptide bonds within a polypeptide chain are classified as **endopeptidases** and those that cleave the peptide bond of either the COOH- or NH_2-terminal amino acid are classified as **exopeptidases.**

Serine proteases often activate other serine proteases from their inactive precursor form, termed a **zymogen,** by the catalytic cleavage of a specific peptide bond in their structure. Serine proteases participate in carefully controlled physiological processes such as blood coagulation (see Clin. Corr. 3.4),

CLINICAL CORRELATION 3.4

Fibrin Formation in a Myocardial Infarct and the Action of Recombinant Tissue Plasminogen Activator (rt-PA)

Coagulation is an enzyme cascade process in which inactive serine protease enzymes (zymogens) are catalytically activated by other serine proteases in a stepwise manner (the coagulation pathway is described in Chapter 22). These multiple activation events generate catalytic products with a dramatic amplification of the initial signal of the pathway. The end product of the coagulation pathway is a cross-linked fibrin clot. The zymogen of the serine protease components of coagulation include factor II (prothrombin) factor VII (proconvertin), factor IX (Christmas factor), factor X (Stuart factor), factor XI (plasma thromboplastin antecedent) and factor XII (Hageman factor). The roman numeral designation was assigned in the order of their discovery and not from their order of action within the pathway. Upon activation of their zymogen forms, the activated enzymes are noted with the suffix "a." Thus prothrombin is denoted as factor II, and the activated enzyme, thrombin, is factor IIa.

The main function of coagulation is to maintain the integrity of the closed circulatory system after blood vessel injury. The process, however, can be dangerously activated in a myocardial infarction and decrease blood flow to heart muscle. About 1.5 million individuals suffer heart attacks each year, resulting in 600,000 deaths. A fibrinolysis pathway also exists in blood to degrade fibrin clots. This pathway also utilizes zymogen factors that are activated to serine proteases. The end reaction is the activation of plasmin, a serine protease. Plasmin acts directly on fibrin to catalyze the degradation of the fibrin clot. Tissue plasminogen activator (t-PA) is one of the plasminogen activators that activates plasminogen to form plasmin. Recombinant t-PA (rt-PA) is produced by gene cloning technology (see Chapter 18). Clinical studies show that the administration of rt-PA shortly after a myocardial infarct significantly enhances recovery. Other plasminogen activators such as urokinase and streptokinase are also effective.

The GUSTO investigators (authors). An international randomized trial comparing tour thrombolytic strategies for acute myocardial infarction. *N. Engl. Med.* 329:673, 1993; International Study Group (authors). In hospital mortality and clinical course of 20,891 patients with suspected acute myocardial infarction randomized between alteplase and streptokinase with or without heparin. *Lancet* 336:71, 1990; and Gillis, J. C., Wagstaff, A. J., and Goa, K. L. Alteplase. A reappraisal of its pharmacological properties and therapeutic use in acute myocardial infarction. *Drugs* 50:102, 1995.

TABLE 3.2 Some Serine Proteases and Their Biochemical and Physiological Roles

Protease	*Action*	*Possible Disease Due to Deficiency or Malfunction*
Plasma kallikrein Factor XIIa Factor XIa Factor IXa Factor VIIa Factor Xa Factor IIa (thrombin) Activated protein C	Coagulation (see Clin. Corr. 3.4)	Cerebral infarction (stroke), coronary infarction, thrombosis, bleeding disorders
Factor $C\bar{1}r$ Factor $C\bar{1}s$ Factor D Factor B C3 convertase	Complement (see Clin. Corr. 3.1)	Inflammation, rheumatoid arthritis, autoimmune disease
Trypsin Chymotrypsin Elastase (pancreatic) Enteropeptidase	Digestion	Pancreatitis
Urokinase plasminogen activator Tissue plasminogen activator Plasmin	Fibrinolysis, cell migreration, embryogenesis, menstruation	Clotting disorders, tumor metastasis (see Clin. Corr. 3.5)
Tissue kallikreins	Hormone activation	
Acrosin	Fertilization	Infertility
α-Subunit of nerve growth factor γ-Subunit of nerve growth factor	Growth factor activation	
Granulocyte elastase Cathepsin G Mast cell chymases Mast cell tryptases	Extracellular protein and peptide degradation, mast cell function	Inflammation, allergic response

fibrinolysis, complement activation (see Clin. Corr. 3.1), fertilization, and hormone production (Table 3.2). The protein activations catalyzed by serine proteases are examples of "limited proteolysis" because only one or two specific peptide bonds of the hundreds in a protein substrate are hydrolyzed. Under denaturing conditions, however, these same enzymes hydrolyze multiple peptide bonds and lead to digestion of peptides, proteins, and even self-digestion (autolysis). Several diseases, such as emphysema, arthritis, thrombosis, cancer metastasis (see Clin. Corr. 3.5), and some forms of hemophilia, are thought to result from the lack of regulation of serine protease activities.

Serine Proteases Exhibit Remarkable Specificity for Site of Peptide Bond Hydrolysis

Many serine proteases exhibit preference for hydrolysis of peptide bonds adjacent to a particular class of amino acid. The serine protease trypsin cleaves following basic amino acids such as arginine and lysine, and chymotrypsin cleaves peptide bonds following large hydrophobic amino acid residues such

CLINICAL CORRELATION 3.5

Involvement of Serine Proteases in Tumor Cell Metastasis

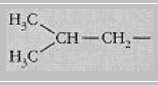
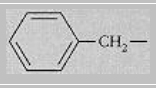
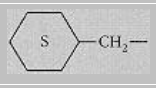
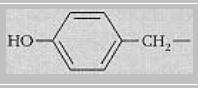
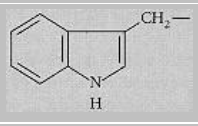
The serine protease urokinase is believed to be required for the metastasis of cancer cells. Metastasis is the process by which a cancer cell leaves a primary tumor and migrates through the blood or lymph system to a new tissue or organ, where a secondary tumor grows. Increased synthesis of urokinase has been correlated with an increased ability to metastasize in many cancers. Urokinase activates plasminogen to form plasmin. Plasminogen is ubiquitously located in extracellular space and its activation to plasmin can cause the catalytic degradation of the proteins in the extracellular matrix through which the metastasizing tumor cells migrate. Plasmin can also activate procollagenase to collagenase, promoting the degradation of collagen in the basement membrane surrounding the capillaries and lymph system. This promotion of proteolytic degradative activity by the urokinase secreted by tumor cells allows the tumor cells to invade the target tissue and form secondary tumor sites.

Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. Plasminogen activators, tissue degradation and cancer. *Adv. Cancer Res.* 44:139, 1985; Yu, H., and Schultz, R. M. Relationship between secreted urokinase plasminogen activator activity and metastatic potential in murine B16 cells transfected with human urokinase sense and antisense genes. *Cancer Res.* 50:7623, 1990; and Fazioli, F., and Blasi, F. Urokinase-type plasminogen activator and its receptor: new targets for anti-metastatic therapy? *Trends Pharmacol. Sci.* 15:25, 1994.

as tryptophan, phenylalanine, tyrosine, and leucine. Elastase cleaves peptide bonds following small hydrophobic residues such as alanine. A serine protease may be called trypsin-like if it prefers to cleave peptide bonds of lysine and arginine, chymotrypsin-like if it prefers aromatic amino acids, and elastase-like if it prefers amino acids with small side chain groups like alanine. The specificity for a certain type of amino acid only indicates its relative preference. Trypsin can also cleave peptide bonds following hydrophobic amino acids, but at a much slower rate than for the basic amino acids. Thus specificity for hydrolysis of the peptide bond of a particular type of amino acid may not be absolute, but may be more accurately described as a range of most likely targets. Each of the identical amino acid hydrolysis sites within a protein substrate is not equally susceptible. Trypsin hydrolyzes each of the multiple arginine peptide bonds in a particular protein at a different catalytic rate, and some may require a conformational change to make them accessible.

Detailed studies of the specificity of serine proteases for a particular peptide bond have been performed with synthetic substrates with fewer than 10 amino acids (Table 3.3). Because these substrates are significantly smaller than the

TABLE 3.3 Reactivity of α-Chymotrypsin and Elastase Toward Substrates of Various Structures

Structure	***Variation in Side Chain Group (Chymotrypsin)***	***Relative Reactivity[a]***
Glycyl	H–	1
Leucyl		1.6×10^4
Methionyl	$CH_3–S–CH_2–CH_2–$	2.4×10^4
Phenylalaninyl		4.3×10^6
Hexahydrophenylalaninyl		8.2×10^6
Tyrosyl		3.7×10^7
Tryptophanyl		4.3×10^7
Variation in chain length (elastase hydrolysis of Ala N-terminal amide)[b]		
	$Ac\text{-}Ala\text{-}NH_2$	
	$Ac\text{-}Pro\text{-}Ala\text{-}NH_2$	1
	$Ac\text{-}Ala\text{-}Pro\text{-}Ala\text{-}NH_2$	1.4×10^1
	$Ac\text{-}Pro\text{-}Ala\text{-}Pro\text{-}Ala\text{-}NH_2$	4.2×10^3
	Ac-Ala-Pro-Ala-Pro-Ala-	4.4×10^5
	NH_2	2.7×10^5

[a] Calculated from values of k_{cat}/K_m found for *N*-acetyl amino acid methyl esters in chymotrypsin substrates.

[b] Calculated from values of k_{cat}/K_m in Thompson, R. C., and Blout, E. R. *Biochemistry* 12:57, 1973.

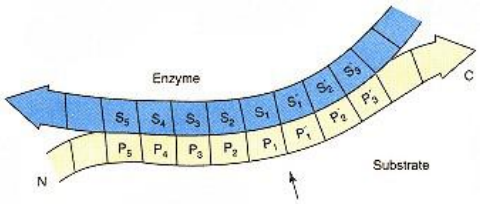


Figure 3.12
Schematic diagram of binding of a polypeptide substrate to binding site in a proteolytic enzyme.
$P_5, P_4, \ldots, P_3'$ are amino acid residues in the substrate that are binding to subsites $S_5, S_4, \ldots, S_3'$ in the enzyme with peptide hydrolysis occurring between P_1–P_1' (arrow). NH_2-terminal direction of substrate polypeptide chain is indicated by N, and COOH-terminal direction by C. Redrawn from Polgar, L. In: A. Neuberger and K. Brocklehurst (Eds.), *Hydrolytic Enzymes*. Amsterdam: Elsevier, 1987, p. 174.

natural ones, they interact only with the catalytic site (primary binding site S_1, see below) and are said to be **active-site directed.** Studies with small substrates and inhibitors indicate that the site of hydrolysis is flanked by approximately four amino acid residues in both directions that bind to the enzyme and impact on the reactivity of the bond hydrolyzed. The two amino acids in the substrate that contribute the hydrolyzable bond are designated S_1—S_1' (Figure 3.13).

Serine Proteases Are Synthesized in a Zymogen Form

Serine proteases are synthesized in an inactive **zymogen** form, which requires limited proteolysis to produce the active enzyme. Those for coagulation are synthesized in liver cells and are secreted into the blood for subsequent activation by other serine proteases following vascular injury. Zymogen forms are usually designated by the suffix -*ogen* after the enzyme name; the zymogen form of trypsin is termed trypsin *ogen* and for chymotrypsin is termed chymotrypsin *ogen*. In some cases the zymogen form is referred to as a **proenzyme;** the zymogen form of thrombin is prothrombin.

Several plasma serine proteases secrete zymogen forms that contain **multiple nonsimilar domains.** Protein C, involved in a fibrinolysis pathway in blood, has four distinct domains (Figure 3.14). The NH_2-terminal domain con-

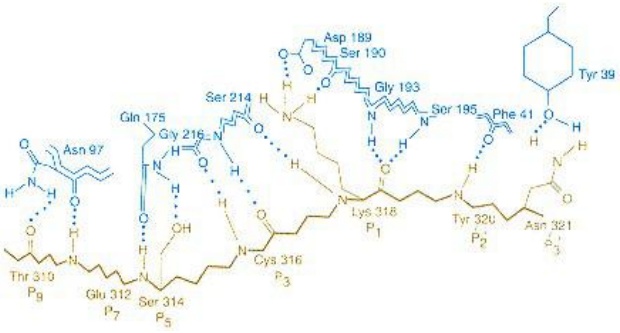


Figure 3.13
Schematic drawing of binding of pancreatic trypsin inhibitor to trypsinogen based on X-ray diffraction data.
Binding-site region of trypsinogen in the complex assumes a conformation like that of active trypsin with inhibitor, which is believed to bind in a similar manner to a substrate in the active enzyme-binding site. One cannot obtain X-ray structures of a natural enzyme–substrate complex because substrate is used up at a rate faster than the time of the X-ray diffraction experiment (see p. 76). Note that inhibitor has an extended conformation so that amino acids $P_9, P_7, P_5, P_3, P_1 \ldots, P_3'$ interact with binding subsites $S_5, \ldots, S_3$. Potentially hydrolyzable bond in inhibitor is between P_1–P_1'
Reprinted with permission from Bolognesi, M., Gatti, B., Menegatti, E., Guarneri, M., Papa-mokos, E., and Huber, *R. J. Mol. Biol*. 162:839, 1983.

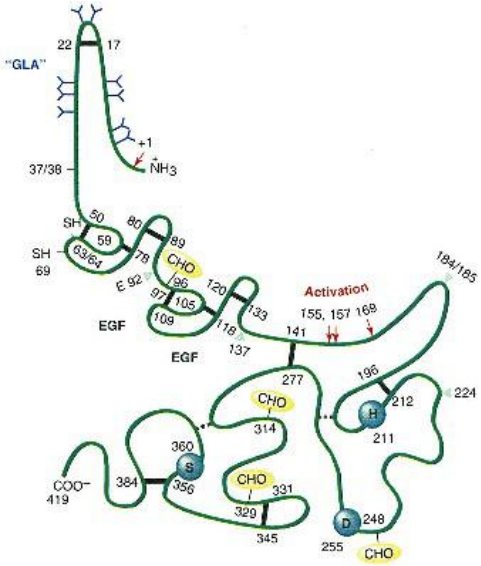


Figure 3.14
Schematic of domain structure for protein C showing multi- domain structure.
"GLA" refers to the -carboxyglutamic residues (indicated by tree structures) in the NH_2-terminal domain, disulfide bridges are indicated by thick bars, EGF indicates positions of epidermal growth factor-like domains, and CHO indicates positions where sugar residues are joined to the polypeptide chain. Proteolytic cleavage sites leading to catalytic activation are shown by arrows. Amino acid sequence is numbered from NH_2-terminal end, and catalytic sites of serine, histidine, and aspartate are shown by circled one-letter abbreviations S, H, and D, respectively.
Redrawn from a figure in Long, G. L. *J. Cell. Biochem.* 33:185, 1987.

tains the derived amino acid, **γ-carboxyglutamic acid** (Figure 3.15), which is enzymatically formed by carboxylation of glutamic acid residues in a vitamin K-dependent reaction. The γ-carboxyglutamic acids chelate calcium ions and form part of a binding site to membranes. The COOH-terminal segment contains the catalytic domains. Activation of these zymogens requires specific proteolysis outside the catalytic domains (Figure 3.14) and is controlled by the binding through the nine γ-carboxyglutamic acid residues at the NH_2-terminal end to a membrane.

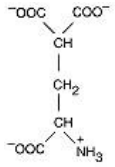


Figure 3.15 Structure of the derived amino acid -carboxy-glutamic acid (abbrev-iation Gla), found in NH_2- terminal domain of many clotting proteins.

There Are Specific Protein Inhibitors of Serine Proteases

Evolutionary selection of this enzyme family for participation in physiological processes requires a parallel evolution of control factors. Specific proteins inhibit the activities of serine proteases after their physiological role has ended (Table 3.4). Thus coagulation is limited to the site of vascular injury and complementation activation leads to lysis only of cells exhibiting foreign antigens. Inability to control these protease systems, which may be caused by a deficiency of a specific inhibitor, can lead to undesirable consequences, such as thrombi formation in myocardial infarction and stroke or uncontrolled reactions of complement in autoimmune disease. Natural inhibitors of serine proteases, termed **serpins** for *ser*ine *p*rotease *in*hibitors, have evolved. This family of inhibitors occurs in animals that have the proteases, but surprisingly these inhibitors are also found in plants that lack proteases.

Serine Proteases Have Similar Structure–Function Relationships

The complex relationships between structure and physiological function in the serine proteases require analysis of a number of observations. (1) Only one serine residue is catalytically active and participates in peptide bond hydrolysis. Bovine trypsin contains 34 serine residues with only one catalytically active or able to react with the inhibitor DFP (see Figure 3.11). (2) X-ray diffraction and amino acid sequence homology studies demonstrate that there are two residues, a histidine and an aspartate, that are always associated with the activated serine

TABLE 3.4 Some Human Proteins that Inhibit Serine Proteases

Inhibitor	*Action*
α_1-Proteinase inhibitor	Inhibits tissue proteases including neutrophil elastase; deficiency leads to pulmonary emphysema
α_1-Antichymotrypsin	Inhibits proteases of chymotrypsin-like specficity from neutrophils, basophils, and mast cells including cathepsin G and chymase
Inter-α-trypsin inhibitor	Inhibits broad range of serine protease activities in plasma
α_2-Antiplasmin	Inhibits plasmin
Antithrombin III	Inhibits thrombin and other coagulation proteases
C_1 Inhibitor	Inhibits complement reaction
α_2-Macroglobulin	General protease inhibitor
Protease nexin I	Inhibits thrombin, urokinase, and plasmin
Protease nexin II	Inhibits growth factor-associated serine proteases, identical to NH_2-terminal domain of amyloid protein secreted in Alzheimer's disease
Plasminogen activator inhibitor I	Inhibits plasminogen activators
Plasminogen activator inhibitor II	Inhibits urokinase plasminogen activator

in the catalytic site. Based on their positions in chymotrypsinogen, these three invariant active site residues of serine proteases are named Ser 195, His 57, and Asp 102. This numbering, based on their sequence number in chymotrypsinogen, is used to identify these residues irrespective of their exact position in the primary structure of any serine protease. (3) Eukaryotic serine proteases exhibit a high sequence and structural similarity with each other. (4) Genes that code for serine proteases are organized similarly (Figure 3.16). In eukaryotic genes, exons are segments of the genomic DNA that are combined into the final messenger RNA that carries the information for the protein. The exons are separated by introns, which are spliced out of RNA and not present in the final messenger RNA (see p. 703). The **exon–intron patterns** of serine proteases show that each of the catalytically essential amino acid residues (Ser 195, His 57, and Asp 102) are on different exons. The catalytically essential histidine and serine are all almost adjacent to their exon boundary. The similarity in exon–intron organization exists for the serine protease family of enzymes among eukaryotic species. The cross-species homology in serine protease gene structure further supports the concept that the serine proteases evolved from a common primordial gene. (5) The catalytic unit of serine proteases exhibits two structural domains, of approximately equal size. The catalytic site is within the interface (crevice) between the two domains. (6) Serine proteases that function through direct interaction with membranes typically have an additional domain to provide this specific function. (7) Natural protein substrates and inhibitors of serine proteases bind through an extended specificity site. (8) Specificity for natural protein inhibitors is marked by extremely tight binding. The binding constant for trypsin to pancreatic trypsin inhibitor is on the order of 10^{13} M^{-1}, reflecting a binding free energy of approximately 18 kcal mol^{-1}. (9) Natural protein inhibitors are usually poor substrates with strong inhibition by the inhibitor requiring hydrolysis of a peptide bond in the inhibitor by the

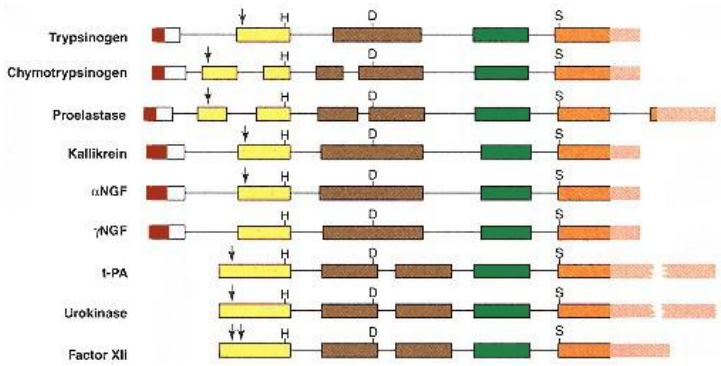


Figure 3.16
Organization of exons and introns in genes that code for serine proteases.
t-PA is tissue plasminogen activator and NGF is nerve growth factor. Exons are shown by boxes and introns by connecting lines. Position of the nucleotide codons for active-site serine, histidine, and aspartate are denoted by S, H, and D, respectively. Red boxes, on left, show regions that code for NH_2-terminal part of polypeptide chain (signal peptide) cleaved before protein is secreted. Light-colored boxes, on right, represent part of gene sequence transcribed into messenger RNA (mRNA), but not translated into protein. Arrows show codons for residues at which proteolytic activation of zymogen forms occurs. Based on a figure in Irwin, D. M., Roberts, K. A., and MacGillivray, R. T. *J. Mol. Biol.* 20:31, 1988.

protease. (10) Serine proteases in eukaryotes are synthesized in zymogen forms to permit their production and transport in an inactive state to their sites of action. (11) Zymogen activation frequently involves hydrolysis by another serine protease. (12) Several serine proteases undergo **autolysis** or self-hydrolysis. Sometimes the self-reaction leads to specific peptide bond cleavage and activation of the catalytic activity. At other times autolysis leads to inactivation of the protease.

Amino Acid Sequence Homology occurs in the Serine Protease Family

Much of our early knowledge of the serine protease family came from trypsin and chymotrypsin purified from bovine materials obtained from a slaughter-house. This has yielded a useful but nonintuitive nomenclature, which uses a sequence alignment against the amino acid sequence of chymotrypsin, to name and number residues of other serine proteases. As mentioned previously, the catalytically essential residues are Ser 195, His 57, and Asp 102. Insertions and deletions of the amino acids in another serine protease are compared to the numbering of residues in chymotrypsin. Alignment is made by algorithms that maximize sequence homology, with exact alignment of the essential serine, histidine, and aspartate residues. These three residues are invariant in all serine proteases and the sequences surrounding them are invariant among the serine proteases of the chymotrypsin family (Table 3.5).

Members of the chymotrypsin family also occur in prokaryotes. Thus bacterial serine proteases from *Streptomyces griseus* and *Myxobacteria* 450 have a structural and functional homology with chymotrypsin. A separate class of serine protease enzymes has been isolated, however, from bacteria that has no structural homology to the mammalian chymotrypsin family. The serine protease subtilisin, isolated from *Bacillus subtilis*, hydrolyzes peptide bonds and contains an activated serine with a histidine and aspartate in its active site but the active

TABLE 3.5 Invariant Sequences Found Around the Catalytically Essential Serine (S) and Histidine (H)

Enzyme	***Sequence (Identical Residues to Chymotrypsin Are in Bold)***																								
	Residues Around Catalytically Essential Histidine																								
Chymotrypsin A	F	**H**	**F**	**C**	**G**	**G**	**S**	**L**	**I**	**N**	**E**	**N**	**W**	**V**	**V**	**T**	**A**	**A**	**H**	**C**	G	V	T	T	S	D
Trypsin	Y	**H**	**F**	**C**	**G**	**G**	**S**	**L**	**I**	**N**	S	Q	**W**	**V**	**V**	S	**A**	**A**	**H**	**C**	Y	K	S	G	I	Q
Pancreatic elastase	A	**H**	T	**C**	**G**	**G**	T	**L**	**I**	R	Q	**N**	**W**	**V**	M	**T**	**A**	**A**	**H**	**C**	V	D	R	E	L	T
Thrombin	E	L	L	**C**	**G**	A	**S**	**L**	**I**	S	D	R	**W**	**V**	L	**T**	**A**	**A**	**H**	**C**	L	L	Y	P	P	W
Factor X	E	G	**F**	**C**	**G**	**G**	T	I	L	**N**	**E**	F	Y	**V**	L	**T**	**A**	**A**	**H**	**C**	L	H	Q	A	K	R
Plasmin	M	**H**	**F**	**C**	**G**	**G**	T	**L**	**I**	S	P	E	**W**	**V**	L	**T**	**A**	**A**	**H**	**C**	L	E	K	S	P	R
Plasma kallikrein	S	F	Q	**C**	**G**	**G**	V	**L**	V	**N**	P	K	**W**	**V**	L	**T**	**A**	**A**	**H**	**C**	K	N	D	N	Y	E
Streptomyces trypsin	-	-	-	**C**	**G**	**G**	A	**L**	Y	A	Q	D	I	**V**	L	**T**	**A**	**A**	**H**	**C**	V	S	G	S	G	N
Subtilisin	V	G	G	A	S	F	V	A	G	E	A	Y	N	T	D	G	N	G	**H**	G	T	H	V	A	G	T
	Residues Around Catalytically Essential Serine																								
Chymotrypsin A	**C**	**A**	**G**	-	-	-	A	S	**G**	**V**	-	-	**S**	**S**	**C**	**M**	**G**	**D**	**S**	**G**	**G**	**P**	**L**	**V**		
Trypsin	**C**	**A**	**G**	Y	-	-	L	E	**G**	G	K	-	D	**S**	**C**	Q	***G***	***D***	***S***	***G***	***G***	***P***	V	**V**		
Pancreatic elastase	**C**	**A**	**G**	-	-	-	G	N	**G**	**V**	R	-	**S**	G	**C**	Q	**G**	**D**	**S**	**G**	**G**	**P**	**L**	**H**		
Thrombin	**C**	**A**	**G**	Y	K	P	G	E	**G**	K	R	G	D	A	**C**	E	**G**	**D**	**S**	**G**	**G**	**P**	F	**V**		
Factor X	**C**	**A**	**G**	Y	-	-	D	T	**Q**	P	E	-	D	A	**C**	Q	**G**	**D**	**S**	**G**	**G**	**P**	H	**V**		
Plasmin	**C**	**A**	**G**	H	-	-	L	A	**G**	G	T	-	D	**S**	**C**	Q	**G**	**D**	**S**	**G**	**G**	**P**	**L**	**V**		
Pl. kallikrein	**C**	**A**	**G**	Y	-	-	L	P	**G**	G	K	-	D	T	**C**	**M**	**G**	**D**	**S**	**G**	**G**	**P**	**L**	I		
Streptomyces trypsin	**C**	**A**	**G**	Y	-	P	D	T	**G**	G	V	-	D	T	**C**	Q	**G**	**D**	**S**	**G**	**G**	**P**	M	F		
Subtilisin	A	G	V	Y	S	T	Y	P	T	N	T	Y	A	T	L	N	**G**	T	**S**	M	A	S	P	H		

Source: From Barrett, A. J. In: A. J. Barrett and G. Salvesen (Eds.), *Proteinase Inhibitors*. Amsterdam: Elsevier, 1986, p. 7.

site arises from structural regions of the protein that bear no sequence or structural homology with the chymotrypsin serine proteases. This serine protease is an example of **convergent evolution** of an enzyme catalytic mechanism. Apparently a gene completely different from those that code for chymotrypsin-like serine proteases evolved the same catalytic mechanism utilizing an active-site serine. The primary and tertiary structure, however, is different from that of the trypsin- and chymotrypsin-like structure.

Tertiary Structures of Serine Proteases Are Similar

Ser 195 in chymotrypsin reacts with diisopropylfluorophosphate (DFP), with a 1:1 enzyme : DFP stoichiometry, that inhibits the enzyme. The three-dimensional structure of chymotrypsin reveals that the Ser 195 is situated within an internal pocket, with access to the solvent interface. His 57 and Asp 102 are oriented so that they participate with the Ser 195 in the catalytic mechanism of the enzyme (see Chapter 4).

Structure determinations by X-ray crystallography have been carried out on many members of this class of proteins (Table 3.6). Structural data are available for catalytically active enzyme forms, zymogens, the same enzyme in multiple species, enzyme–inhibitor complexes, and a particular enzyme at different temperatures and in different solvents. The most complete analysis has been that of trypsin. Its X-ray diffraction analysis has yielded a three-dimensional structure at better than 1.7-Å resolution, which can resolve atoms at a separation of 1.3 Å such as the C=0 separation of the carbonyl group (1.2 Å). This resolution, however, is not uniform over the entire trypsin structure. Different regions of the molecule have a variable tendency to be localized in space during the time course of the X-ray diffraction experiment, and for some atoms in the structure their exact position cannot be as precisely defined as for others. The structural disorder is especially apparent in surface residues not in contact with neighboring molecules. Rapid methods for X-ray data acquisition (see Chapter 2) further support this observation of dynamic fluctuation. Trypsin is globular in its overall shape and consists of two domains of approximately equal size (Figure 3.17), which do not penetrate one another. The secondary structure of trypsin has little α-helix, except in the COOH-terminal region of the molecule. The structure is predominantly β-structure, with each of the

TABLE 3.6 Serine Protease Structures Determined by X-Ray Crystallography

Enzyme	*Species Source*	*Inhibitors Present*	*Resolution (Å)*
Chymotrypsin[a]	Bovine	Yes[b]	1.67[c]
Chymotrypsinogen	Bovine	No	2.5
Elastase	Porcine	Yes	2.5
Kallikrein	Porcine	Yes	2.05
Proteinase A	*S. griseus*	No	1.5
Proteinase B	*S. griseus*	Yes	1.8
Proteinase II	Rat	No	1.9
Trypsin[a]	Bovine	Yes[b]	1.4[c]
Trypsinogen[a]	Bovine	Yes[b]	1.65[c]

[a] Structure of this enzyme molecule independently determined by two or more investigators.

[b] Structure obtained with no inhibitor present (native structure) and with inhibitors. Inhibitors used include low molecular weight inhibitors (i.e., benzamidine, DFP, and tosyl) and protein inhibitors (i.e., bovine pancreatic trypsin inhibitor).

[c] Highest resolution for this molecule of the multiple determinations.

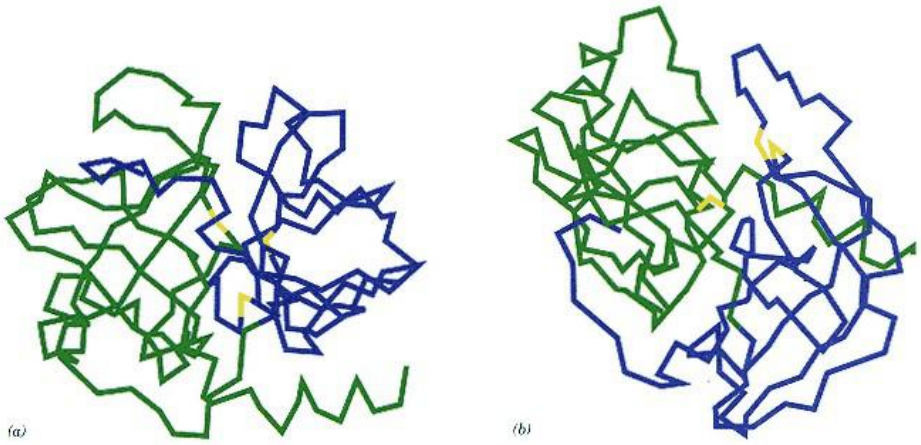


Figure 3.17
Two views of the structure of trypsin showing tertiary structure of two domains.
Active-site serine, histidine, and aspartate are indicated in yellow.

domains in a "deformed" β-barrel. Loop regions protrude from the barrel ends, being almost symmetrically presented by each of the two folded domains. These loop structures combine to form a surface region of the enzyme that extends outward, above the catalytic site. These loops have a structural and functional similarity to the CDRs of immunoglobulins.

Alignment of three-dimensional structures can be performed on serine proteases using a mathematical function that compares structural equivalence and allows for insertion and deletion of amino acids in a particular sequence. The data of Table 3.7 contrast the extent of structural superimposability with the homology of sequences brought into coincidence by the structural superposition. This table shows the total number of amino acids and the number that are statistically identical in each structure, by X-ray diffraction, in their topological position, even if they are chemically different amino acids. Topologically equivalent amino acids have the same relationship in three-dimensional space to the point where they cannot be distinguished from one another by X-ray diffraction. The last column presents the number of amino acids that are chemically identical. In these structural alignments the regions of greatest difference appear to be localized to the CDR-like loop regions, which extend from the β-barrel domains to form the surface region out from the catalytic site. The effect of

TABLE 3.7 Structural Superposition of Selected Serine Proteases and the Resultant Amino Acid Sequence Comparison

Comparison	*Number of Amino Acids in Sequence*		*Number of Structurally Equivalent Residues*	*Number of Chemically Identical Residues*
	Protease 1	*Protease 2*		
Trypsin-elastase	223	240	188	81
Trypsin-chymotrypsin	223	241	185	93
Trypsin-mast cell protease	223	224	188	69
Trypsin-prekallikrein	223	232	194	84
Trypsin-*S. griseus* protease	223	180	121	25

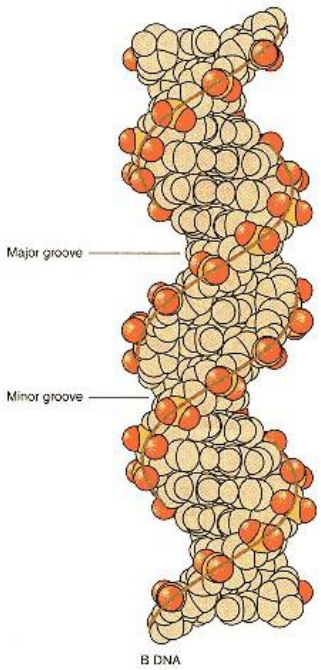


Figure 3.18
Space-filling model of DNA in B conformation showing major and minor grooves.
Reprinted with permission from Rich, A. *J. Biomol. Struct. Dyn.* 1:1, 1983.

altering the amino acids in these loops is to alter the **macromolecular binding specificity** of the protease. It is the structure of the loop in factor Xa, for example, that allows it to specifically bind to prothrombin. Serpins interact with different proteases based on their affinity for the loop structures. Bacterial proteases related to the eukaryotic serine protease family contain the same two domains as do the eukaryotic family but lack most of the loop structures. This agrees with the lack of a requirement of bacterial proteases for complex interactions that the eukaryotic protease must carry out and the observation that bacterial proteases are not produced in a zymogen form.

Thus the serine protease family constitutes a structurally related series of proteins that use a catalytically active serine. During evolution, the basic two-domain structure and the catalytically essential residues have been maintained, but the region of the secondary interactions (loop regions) have changed to give the different proteins of the family their different specificities toward substrates, activators, and inhibitors, characteristic of their important physiological functions.

3.4— DNA-Binding Proteins

Regulatory sites exist in DNA that bind proteins that control gene expression. These sites contain a nucleotide sequence that binds regulatory proteins known as transcription factors. The specific DNA sequence, or **transcription factor binding element**, is usually less than 10 nucleotides long. Noncovalent interactions between the protein and DNA allow the protein to recognize the nucleotide sequence and bind to a specific regulatory site. This is a highly selective feat as the human genome has up to 100,000 genes, each with its own regulatory sequences. While there are huge gaps in our knowledge of how proteins regulate gene expression, some common structural motifs of DNA-binding proteins are apparent.

Three Major Structural Motifs of DNA-Binding Proteins

Along the helical spiral of a DNA molecule in its most common form (B form) are two grooves, the major and minor grooves (Figure 3.18) (see Chapter 14) to which the proteins must associate. A structural motif found in many DNA-binding proteins is the **helix–turn–helix (HTH).** An HTH places one of its α-helices, designated the **recognition helix**, across the major groove where side chain residues of the helix form specific noncovalent interactions with the base sequence of the target DNA. The interaction appears to induce distortions in conformation of the B-DNA binding site that better accommodate the interactions with protein. Nonspecific interactions are made between the protein and sugar–phosphate backbone of DNA. HTH proteins bind as dimers; thus there are two helix–turn–helix motifs per active regulatory protein. X-ray structures show the two helix–turn–helix motifs protruding from the structure of each monomer domain binding at two adjacent turns of the major groove in the DNA, making a strong protein–DNA interaction (Figures 3.19–3.21).

The **zinc-finger** motif is another structure found in some DNA-binding proteins. Zinc-finger proteins contain repeating motifs of a Zn^{2+} atom bonded to two cysteine and two histidine side chains (Figure 3.22). In some cases the histidines may be substituted by cysteines. The primary structure for the motif contains two close cysteines separated by about 12 amino acids from a second pair of Zn^{2+} liganding amino acids (histidine or cysteine). The three-dimensional structure of one zinc finger has been deduced by ^{1}H-NMR (Figure 3.23). The motif contains an α-helix segment that can bind within the major groove at its target site in DNA and makes specific interactions with the nucleotide base sequence.

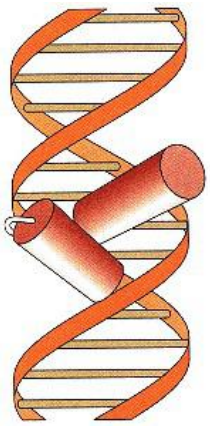


Figure 3.19
Binding of a helix–turn –helix motif into the major groove of B-DNA.
The recognition helix lies across the major groove.
Redrawn from Schleif, *R. Science* 241: 241, 1988.

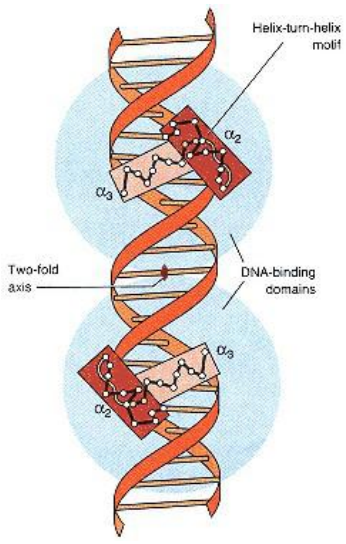


Figure 3.20
Association of a DNA-binding protein (dimer) with two helix–turn–helix motifs into adjacent major grooves of B-DNA.
Redrawn from Brennan, R. G., and *Matthews, B. W. Trends Biochem. Sci.* 14:287, 1989 (Fig. 1b).

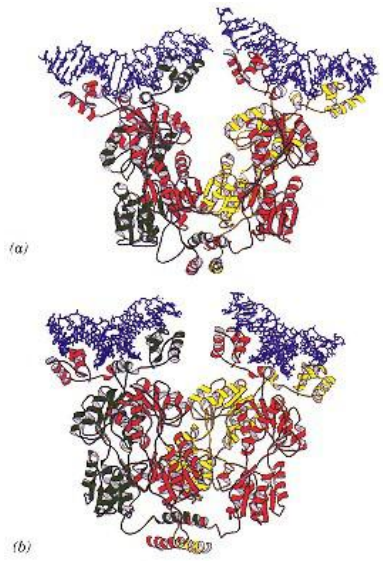


Figure 3.21
X-ray crystallographic structure of helix–turn–helix motif *lac* repressor protein in association with target DNA.
(a) Repressor is a tetramer protein with individual monomers colored green and violet (left), red and yellow (right). The DNA targets are colored blue (top). Recognition helices from dimer of tetramer are shown to interact in adjacent major grooves of target DNAs. Each dimer in tetramer interacts with a discrete (separated) target consensus sequence present in DNA.
(b) A different view of the same tetramer.
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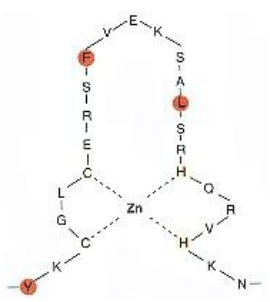


Figure 3.22
Primary sequence of a zinc-finger motif found in DNA-binding protein Xfin from *Xenopus*.
Invariant and highly conserved amino acids in structure are circled in dark red.
Redrawn from Lee, M. S., Gippert, G. P., Soman, K. V., Case, D. A., and Wright, P. E. *Science*245:635, 1989.

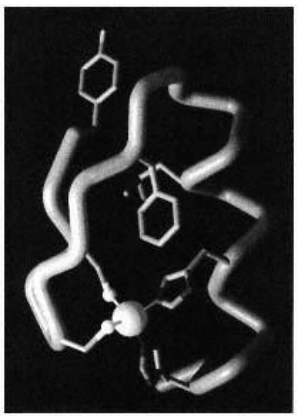
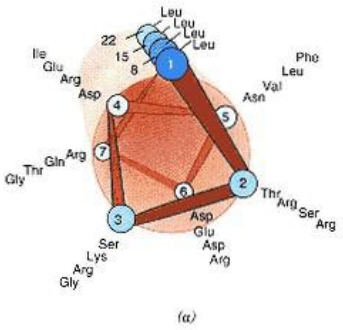


Figure 3.23
Three-dimensional structure obtained by ^{1}H-NMR of zinc-finger motif from *Xenopus* protein Xfin (sequence shown in Figure 3.22).
Superposition of 37 possible structures derived from calculations based on the ^{1}H-NMR. NH_2 terminal is at upper left and COOH terminal is at bottom right. Zinc is sphere at the bottom with Cys residues to the left and His residues to the right. Photograph provided by Michael Pique, and Peter E. Wright, Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California.



(a)

(b)

Figure 3.24
Leucine zipper motif of DNA-binding proteins.
(a) Helical wheel analysis of the leucine-zipper motif in DNA enhancer-binding protein. The amino acid sequence in the wheel analysis is displayed end-to-end down the axis of a schematic -helix structure. The leucines (Leu) are observed in alignment along one edge of the helix (residues 1, 8, 15, and 22 in the sequence).
(b) The X-ray structure, in side view, in which the helices are presented in ribbon form and side chains in stick form. Contacting leucine residues in yellow and green.
(a) Redrawn from Landschulz, W. H., Johnson, P. F., and McKnight, S. L. *Science* 240:1759, 1988.
(b) Figure reproduced with permission from D. Voet and J. Voet, *Biochemistry, 2nd ed*. New York: Wiley, 1995 and based on an X-ray structure by Peter Kim, MIT, and Tom Alber, University of Utah School of Medicine.

A third structural motif found in some of the DNA-binding proteins is the **leucine zipper**. Leucine zippers are formed from a region of α-helix that contain at least four leucines, each leucine separated by six amino acids from one another (i.e., Leu-X_6-Leu-X_6-Leu-X_6-Leu, where X is any common amino acid). With 3.6 residues per turn of the α-helix, the leucines align on one edge of the helix, with a leucine at every second turn of the helix (Figure 3.24). The leucine-rich helix forms a hydrophobic interaction with a second leucine helix on another polypeptide chain subunit, to "zipper" the two subunits together to form a dimer (Figure 3.25). The leucine-zipper motif does not directly interact with the DNA, as do the zinc-finger or helix–turn–helix motifs. Mutations in the zipper motif show that if the dimer is not formed by association of the monomers through the zipper, the protein will not bind to DNA strongly. However, just adjacent to the α-helix of the zipper motif in the primary structures

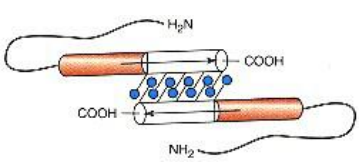


Figure 3.25
Schematic diagram of two proteins with leucine zippers in antiparallel association.
DNA-binding domains containing a high content of basic amino acids (arginines and lysines) are shown in pink.
Redrawn from Landschulz, W. H., Johnson, P. F., and McKnight, S. L. *Science* 240:1759, 1988.

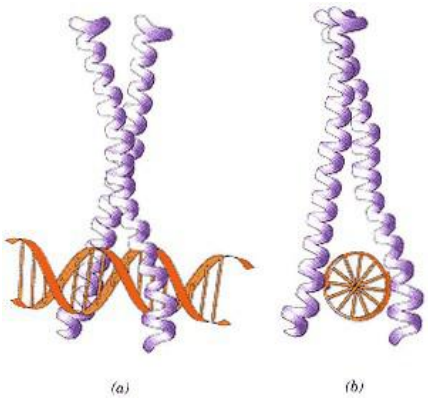


Figure 3.26
Structure of the bZIP GCN4–DNA complex.
(a) bZIP protein is a dimer (polypeptide chains colored blue) with each monomer joined by a leucine-zipper motif. NH_2 termini diverge to allow the basic region of the sequence to interact in the major groove of DNA target site (DNA colored red). (b) Same interaction viewed down the DNA axis. From Ellenberger, T. E., Brandl, C. J., Struhl, K., and Harrison, S. C. *Cell* 71:1223, 1992.

there is a sequence containing a high concentration of basic amino acids, arginine and lysine. This evolutionary conserved basic region interacts with the DNA. The positive charges of the arginine and lysine side chains are drawn to the negatively charged DNA phosphate groups.

The yeast transcription factor GCN4 is one eukaryotic DNA-binding protein that contains the leucine-zipper (bZIP) motif. It is a dimer of two continuous α-helical subunits joined by a leucine-zipper interface. The α-helices cross at this interface and then diverge with their two N-terminal ends separated to pass directly through different sides of the same major groove of the DNA target site (Figure 3.26). Amazingly, there are no bends or kinks in the linear helical structure of each subunit of the dimer. As discussed above, the DNA contact regions contain many positively charged amino acid residues that interact with negatively charged phosphate groups in the DNA.

Many regulatory proteins with the leucine-zipper motif have been shown to be oncogene products (Myc, Jun, and Fos). Fos forms a heterodimer with Jun through a leucine-zipper interaction, and the Fos/Jun dimers bind to gene regulatory sites. If these regulatory proteins are mutated or produced in an unregulated manner, the cell can be transformed to a cancer cell.

DNA-Binding Proteins Utilize a Variety of Strategies for Interaction with DNA

The helix–loop–helix motif was the first motif to be identified for interaction with DNA. X-ray structural studies of protein–DNA complexes show a great variety of other mechanisms for protein–DNA association. The TATA box-binding protein (TBP) associates with the TATA sequence of gene promoters. Association of TBP with the TATA sequence forms the foundation for a large protein complex that initiates gene transcription by RNA polymerase. The X-ray structure of the C-terminal domain of the TBP bound to a TATA sequence shows that TBP contains two domains, each composed of a curved antiparallel β-sheet with a concave surface. The two-domain structure forms the shape of a "saddle" that sits over the DNA double helix. The concave surface of the "saddle" distorts the B-DNA structure and partially unwinds the DNA helix. This distortion, in turn, produces a wide open, though shallow, minor groove that interacts extensively with the under portion of the TBP saddle (Figure 3.27*a*). One critical protein that forms a part of the initiation complex for RNA transcription is TFIIB. An X-ray structure shows TFIIB associates with one of the "stirrups" of the TBP "saddle" in the TATA sequence complex (Figure 3.27*b*).

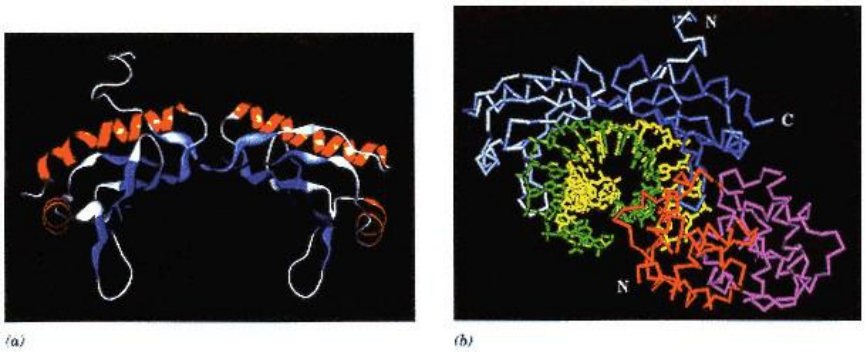


Figure 3.27
Structures of TBP–DNA binary and TBP–TFIIB–DNA ternary complexes.
(a) Computer model generated from X-ray structure of TBP interaction with DNA; -helices and -strands are shown in red and blue, respectively, with the remainder in white, (b) TBP–TFIIB–DNA complex. Proteins are depicted as -carbon traces while the DNA is shown as an atomic stick model. TFIIB first repeat is colored red and the second repeat magenta. One domain of TBP is light blue while the second is dark blue. DNA-coding strand is colored green and noncoding strand is in yellow. N and C termini of TBP and TFIIB are labeled when visible.
Courtesy of S. K. Burley.
Reprinted with permission from (a) Nikolov, D. B., Hu, S.-H., Lin, J., Gasch, A., Hoffmann, A., Horikoshi, M., Chua, N.-H., Roeder, R. G., and Burley, S. K. *Nature* 360:40, 1992; and (b) Nikolov, D. B., Chen, H., Halay, E. D., Usheva, A. A., Hisatake, K., Lee, D. K., Roeder, R. G., and Burley, S. K. *Nature* 377:119, 1995.

The p53 protein is a transcription factor that, on sensing damaged DNA, upregulates the expression of genes that inhibit cell division, giving the cell time to repair the damaged DNA. Alternatively, it can instruct the cell to undergo apoptosis (programmed cell death) if the DNA damage is too extensive for repair. This transcription factor is a key tumor suppressor protein and mutant forms of p53 are found in the majority of human cancers. The DNA-binding domain of p53 consists of two sheets of antiparallel β-strands like an immuno-globulin fold. This central fold provides the scaffolding for the **loop–sheet–helix motif** and for the two large loops (15 and 32 residues) that interact with the DNA. The α-helix (designated H2) of the loop–sheet–helix motif fits into a major groove with loop 1 (L1), while loop 3 (L3) interacts strongly with the adjacent minor groove (Figure 3.28*a*). Figure 3.28*b* shows the side chains of the amino acids commonly found mutated in human cancers. Many mutations are in residues that interact directly with the DNA, such as Arg 248, which is a part of loop 3. Other common mutations are in residues within the domain core required for protein stability, p53 binds as a tetramer to DNA (Figure 3.28*c*).

NF-κB transcription factors are ubiquitous transcription factors of the Rel family. They regulate a variety of genes, especially genes with roles in cellular defense mechanisms against infection and in differentiation. The NF-κB p50 protein has two domains interconnected by a 10 amino acid linker region (Figure 3.29*a*). Each domain contains a β-barrel core with antiparallel strands that have structural homology to the immunoglobulin fold motif. The C-terminal domains provide the dimer interface, in which one surface of each immunoglobulin fold pack together to form the subunit interface. Both N-terminal and C-terminal domains, as well as the loop that connects them, bind to the DNA surface, contributing 10 loops (5 from each subunit in the dimer) that fill the entire major groove in the target DNA (Figure 3.29). N-terminal domains also have an α-helical segment that forms a strong interaction in the minor groove near the center of the target element. In contrast to many other DNA-binding proteins, the NF-κB p50 dimer does not make contact with two separated sites

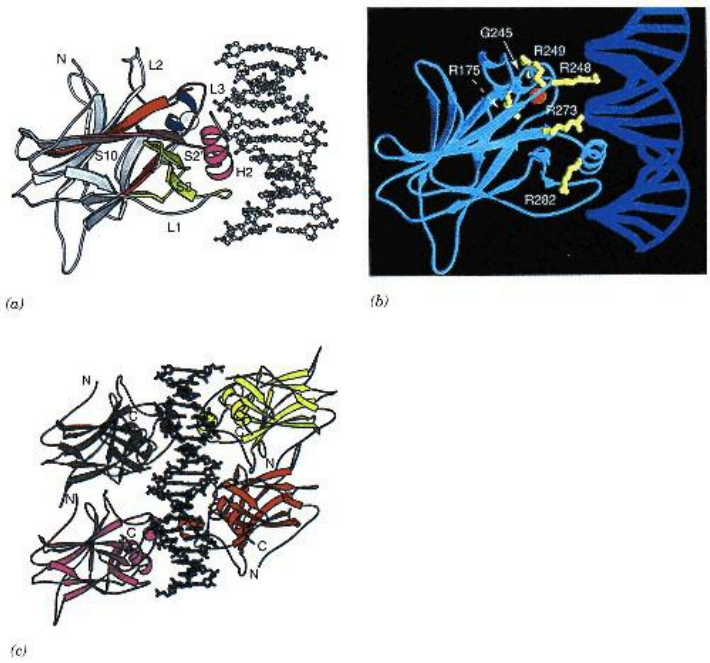


Figure 3.28
Structure of p53–DNA complex.
(a) Structure of p53 core domain complexed with DNA. -Strands (S), -helices (H), loops (L), and zinc atom (sphere) are lettered and numbered. Helix (H2), loop 1 (L1), and loop 3 (L3) associate in major and minor grooves of target DNA.
(b) Frequently mutated amino acid side chains commonly found in human cancers are colored yellow. Zinc atom is colored red.
(c) Structure of tetramer p53 in association with DNA. Each monomer of tetramer binds to a discrete consensus binding site in the target DNA. Four core domains of the tetramer are colored green, purple, yellow, and red-brown, and DNA is colored blue.
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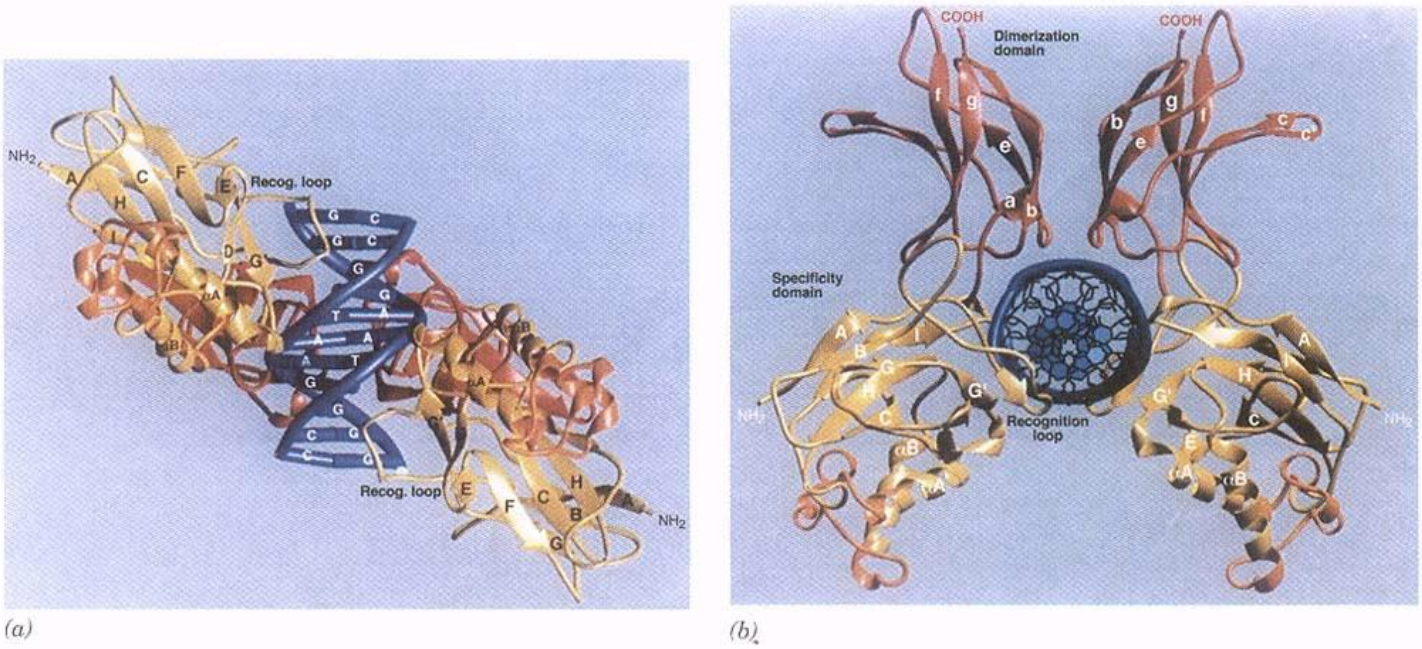


Figure 3.29
Structure of the NF-κB p50 homodimer to DNA.
Only residues 43 through 352 of both subunits are shown in structures. NF- B p50 protein binds as a dimer. In each monomer, the N-terminal domain is colored yellow and the C-terminal domain is colored red-brown. Orange insert in N-terminal domain is a region unique to p50 and not present in other structures of Rel family of transcription factors.
(a) View along DNA axis.
(b) Alternative view of same complex.
Reprinted with permission from Müller, C. W., Rey, F. A., Sodeoka, M., Verdine, G. L., and Harrison, S. C. *Nature* 373:311, 1995.

on the DNA target. Rather, the contacts from one monomer combine with those of the second monomer to form a continuous interaction through the single binding site in the DNA.

3.5— Hemoglobin and Myoglobin

Hemoglobins are globular proteins, present in high concentrations in red blood cells, that bind oxygen in the lungs and transport the oxygen in blood to tissues and cells around the capillary beds of the vascular system. Hemoglobins also transport carbon dioxide and protons from the tissues to the lungs. Hemoglobins carry and release nitric oxide (NO), a potent vasodilator and inhibitor of platelet aggregation (see p. 995). In this section the structural and molecular aspects of hemoglobin and myoglobin are described. The physiological roles of these proteins are discussed in Chapter 25.

Human Hemoglobin Occurs in Several Forms

A hemoglobin molecule consists of four polypeptide chains, two each of two different amino acid sequences. The major form of human adult hemoglobin, **HbA_1,** consists of two α chains and two β chains ($\alpha_2\beta_2$). The α polypeptide has 141 and the β polypeptide has 146 amino acids. Other forms of hemoglobin predominate in the blood of the human fetus and early embryo (Figure 3.30). The fetal form (**HbF**) contains the same α chains found in HbA_1, but a second type of chain (γ chain) occurs in the tetramer molecule and differs in amino acid sequence from that of the β chain of adult HbA_1 (Table 3.8). Additional forms appear in the first months after conception (embryonic) in which the α chains are substituted by *zeta* (ζ) chains of different amino acid sequence and the ε chains serve as the β chains. A minor form of adult hemoglobin, HbA_2, comprises about 2% of normal adult hemoglobin and contains two α chains and two chains designated delta (δ) (Table 3.8).

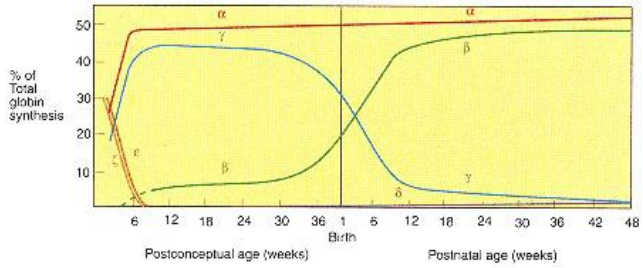


Figure 3.30
Changes in globin chain production during development.
Based on a figure in Nienhuis, A. W. and Maniatis, T. In: G. Stamatoyannopoulos, A. W. Nienhuis, P. Leder, and P. W. Majerus (Eds), *The Molecular Basis of Blood Diseases*. Philadelphia: Saunders, 1987, p. 68, in which reference of Weatherall, D. J., and Clegg, J. B., *The Thalassemia Syndromes*, 3rd ed. Oxford: Blackwell Scientific Publications, 1981, is acknowledged.

TABLE 3.8 Chains of Human Hemoglobin

Developmental Stage	*Symbol*	*Chain Designations*
Adult	HbA_1	$\alpha_2\beta_2$
Adult	HbA_2	$\alpha_2\delta_2$
Fetus	HbF	$\alpha_2\gamma_2$
Embryo	Hb Gower-1	$\zeta_2\varepsilon_2$
Embryo	Hb Portland	$\zeta_2\gamma_2$

Myoglobin: A Single Polypeptide Chain with One O_2-Binding Site

Myoglobin (Mb) is an O_2-carrying protein that binds and releases O_2 with changes in the oxygen concentration in the sarcoplasm of skeletal muscle cells. In contrast to hemoglobin, which has four polypeptide chains and four O_2-binding sites, myoglobin contains only a single polypeptide chain and one O_2-binding site. Myoglobin is a model for what occurs when a single protomer molecule acts alone without the interactions exhibited among the four O_2-binding sites in the more complex tetramer molecule of hemoglobin.

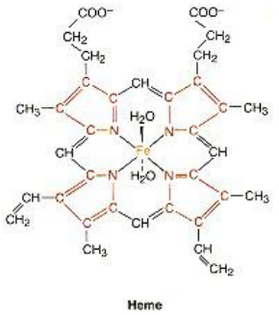


Figure 3.31
Structure of heme.

A Heme Prosthetic Group Is at the Site of O_2 Binding

The four polypeptides of globin subunits in hemoglobin and the one of myoglobin each contain a heme prosthetic group. A **prosthetic group** is a nonpolypeptide moiety that forms a functional part of a protein. Without its prosthetic group, a protein is designated an **apoprotein**. With its prosthetic group it is a **holoprotein**.

Heme contains protoporphyrin IX (see Chapter 24) with an iron atom in its center (Figure 3.31). The iron atom is in the ferrous (2+ charge) oxidation state in functional hemoglobin and myoglobin. The ferrous atom in the heme can form five or six ligand bonds, depending on whether or not O_2 is bound to the molecule. Four bonds are to the pyrrole nitrogen atoms of the porphyrin. Since all pyrrole rings of porphyrin lie in a common plane, the four ligand bonds from the porphyrin to the iron atom will have a tendency to lie in the plane of the porphyrin ring. The fifth and the potentially sixth ligand bonds to the ferrous atom are directed along an axis perpendicular to the plane of the porphyrin ring (Figure 3.32). The fifth coordinate bond of the ferrous atom is to a nitrogen of a histidine imidazole. This is designated the **proximal histidine** in hemoglobin and myoglobin structures (Figures 3.32 and 3.33). O_2 forms a sixth coordinate bond to the ferrous atom when bound to hemoglobin. In this bonded position the O_2 is placed between the ferrous atom to which it is liganded and a second histidine imidazole, designated the **distal histidine**. In deoxyhemoglobin, the sixth coordination position of the ferrous atom is unoccupied.

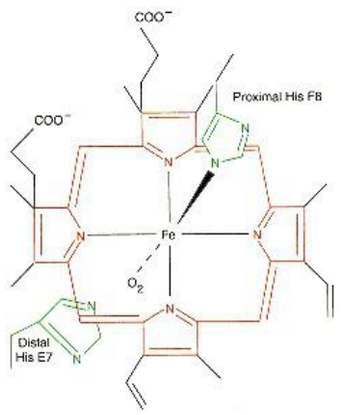


Figure 3.32
Ligand bonds to ferrous atom in oxyhemoglobin.

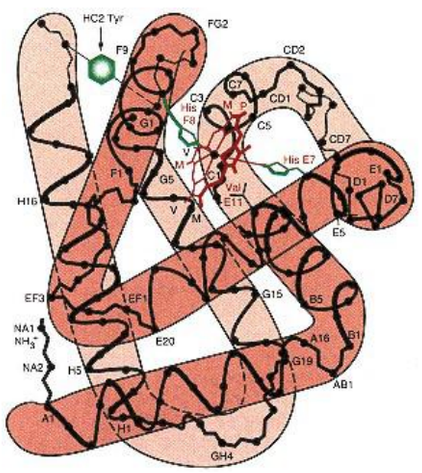


Figure 3.33
Secondary and tertiary structure characteristics of chains of hemoglobin.
Proximal His F8, distal His E7, and Val E11 side chains are shown. Other amino acids of polypeptide chain are represented by -carbon positions only; the letters M, V, and P refer to the methyl, vinyl, and propionate side chains of the heme. Reprinted with permission from Perutz, M. *Br. Med. Bull.* 32: 195, 1976.

The porphyrin part of the heme is positioned within a hydrophobic pocket of each globin subunit. In the heme pocket X-ray diffraction studies show that approximately 80 interactions are provided by approximately 18 residues to the heme. Most of these noncovalent interactions are between apolar side chains of amino acids and the apolar regions of the porphyrin. As discussed in Chapter 2, the driving force for these interactions is the expulsion of water of solvation on association of the hydrophobic heme with the apolar amino acid side chains in the heme pocket. In myoglobin additional noncovalent interactions are made between the negatively charged propionate groups of the heme and positively charged arginine and histidine side chains of the protein. However, in hemoglobin chains a difference in the amino acid sequence in this region of the heme-binding site leads to stabilization of the porphyrin propionates by interaction with an uncharged histidine imidazole and with water molecules of solvent toward the outer surface of the molecule.

X-Ray Crystallography Has Assisted in Defining the Structure of Hemoglobin and Myoglobin

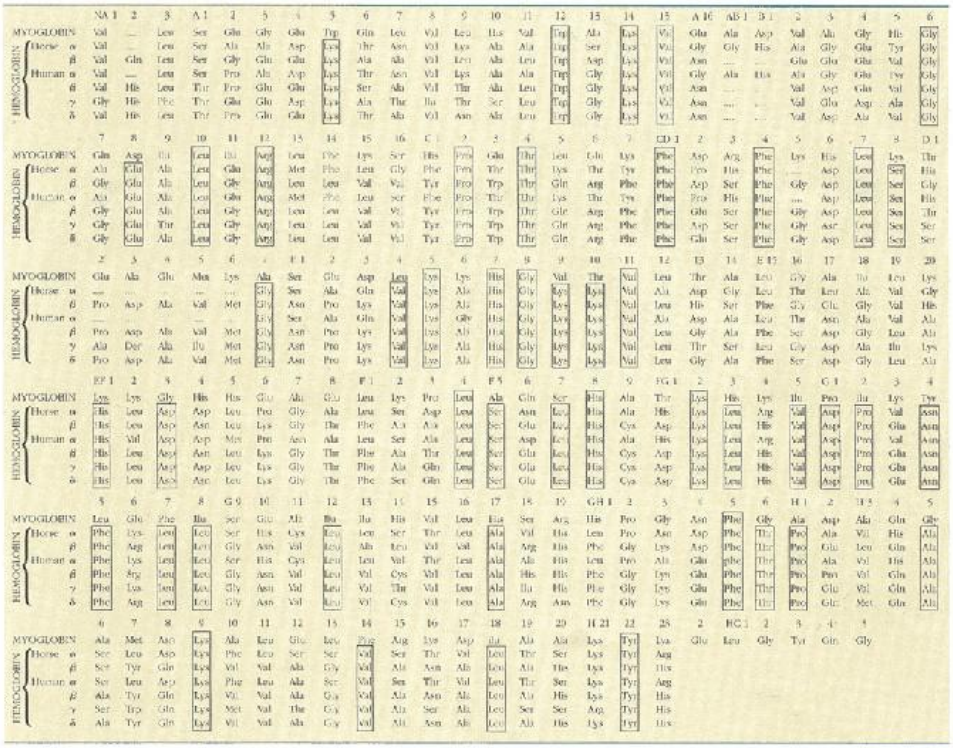
The structure of deoxy and oxy forms of hemoglobin and myoglobin have been resolved by X-ray crystallography. In fact, sperm whale myoglobin was the first globular protein whose full three-dimensional structure was determined by this technique. This was followed by the X-ray structure of the more complex horse hemoglobin molecule. These structures show that each globin polypeptide in the hemoglobins and the single subunit of myoglobin are composed of multiple α-helical regions connected by turns of the polypeptide chain that allow the protein to fold into a spheroidal shape (Figure 3.33). The mechanism of cooperative associations of O_2, discussed below, is based on the X-ray structures of oxyhemoglobin, deoxyhemoglobin, and a variety of hemoglobin derivatives.

Primary, Secondary, and Tertiary Structures of Myoglobin and the Individual Hemoglobin Chains

The amino acid sequences of the polypeptide chain of myoglobin of 23 different animal species have been determined. All myoglobins contain 153 amino acids in their polypeptide chains, of which 83 are invariant. Only 15 of these invariant residues in the myoglobin sequence are identical to the invariant residues of the sequenced mammalian globins of hemoglobin. However, the changes are, in the great majority of cases, conservative and preserve the general physical properties of the residues (Table 3.9). Since myoglobin is active as a monomer, many of its surface positions interact with water and prevent another molecule of myoglobin from associating. In contrast, surface residues of the individual subunits in hemoglobin are designed to provide hydrogen bonds and nonpolar contacts with other subunits in the hemoglobin quaternary structure. Proximal and distal histidines are preserved in the sequences of all the polypeptide chains. Other invariant residues are in the hydrophobic heme pocket and form essential nonpolar contacts with the heme that stabilize the heme–protein complex.

While there is surprising variability in amino acid sequences among the different polypeptide chains, to a first approximation the secondary and tertiary structures of each of the subunits of hemoglobin and myoglobin are almost identical (Figure 3.34). Significant differences in physiological properties between α, β, γ, and δ chains of hemoglobins and the polypeptide chain of myoglobin are due to rather small specific changes in their structures. The similarity in tertiary structure, resulting from widely varied amino acid sequences, shows that the same tertiary structure for a protein can be arrived at by many different sequences.

TABLE 3.9 Amino Acid Sequences of Human Hemoglobin Chains and of Sperm Whale Myoglobin[a]



Source: Based on diagram in Dickerson, R. E., and Geis, I. *The Structure and Function of Proteins.* New York: Harper & Row, 1969, p. 52.

[a] Residues that are identical are enclosed in box. A, B, C, . . . , designate different helices of tertiary structure (see text).

Approximately 70% of the residues participate in the α-helical secondary structures generating seven helical segments in the β chain and eight in the β chain. These latter eight helical regions are commonly lettered A–H, starting from the first (A) helix at the NH_2-terminal end. The interhelical regions are designated as AB, BC, CD, . . . , GH, respectively. The nonhelical region between the NH_2-terminal end and the A helix is designated the NA region; and the region between the COOH-terminal end and the H helix is designated the HC region (Figure 3.33). This naming system allows discussion of particular residues that have similar functional and structural roles in hemoglobin and myoglobin.

A Simple Equilibrium Defines O_2 Binding to Myoglobin

The association of oxygen to myoglobin is characterized by a simple equilibrium constant (Eqs. 3.1 and 3.2) In Eq. 3.2 $[MbO_2]$ is the solution concentration of oxymyoglobin, [Mb] is that of deoxymyoglobin, and $[O_2]$ is the concentration

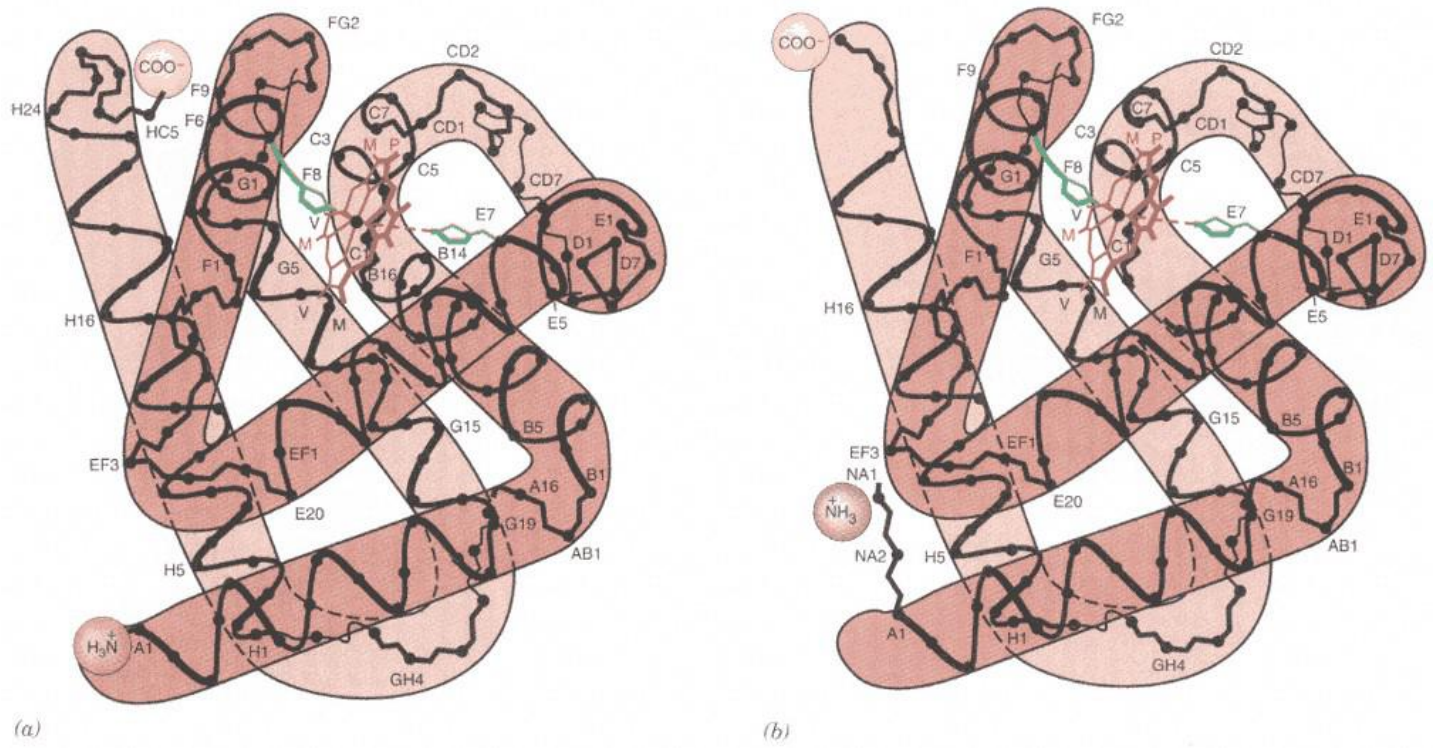


Figure 3.34
Comparison of conformation of
(a) myoglobin and
(b) β chain of HbA_1.

Overall structures are very similar, except at NH_2-terminal and COOH-terminal ends.
Reprinted with permission from Fersht,
A. *Enzyme Structure and Mechanism*. San Francisco: Freeman, 1977, pp. 12, 13.

of oxygen, in moles per liter. The equilibrium constant, K_{eq}, will also have the units of moles per liter. As for any true equilibrium constant, the value of K_{eq} is dependent on pH, ionic strength, and temperature.

$$Mb + O_2 \overset{K_{eq}}{\rightleftharpoons} MbO_2 \qquad (3.1)$$

$$K_{eq} = \frac{[Mb][O_2]}{[MbO_2]} \qquad (3.2)$$

Since oxygen is a gas, it is more convenient to express its concentration as the pressure of oxygen in torr (1 torr is equal to the pressure of 1 mmHg at 0°C and standard gravity). In Eq. 3.3 this conversion of units has been made: P_{50}, the equilibrium constant, and pO_2, the concentration of oxygen, being expressed in torr.

$$P_{50} = \frac{[Mb] \cdot pO_2}{[MbO_2]} \qquad (3.3)$$

An oxygen-saturation curve characterizes the properties of an oxygen-binding protein. In this plot the fraction of oxygen-binding sites in solution that contain oxygen (Y, Eq. 3.4) is plotted on the ordinate *versus* pO_2 (oxygen concentration) on the abscissa. The Y value is simply defined for myoglobin by Eq. 3.5. Substitution into Eq. 3.5 of the value of $[MbO_2]$ obtained from Eq. 3.3, and then dividing through by [Mb], results in Eq. 3.6, which shows the dependence of Y on the value of the equilibrium constant P_{50} and the oxygen concentration. It is seen from Eqs. 3.3 and 3.6 that the value of P_{50} is equal to the oxygen concentration, pO_2, when $Y = 0.5$ (50% of the available sites occupied)–hence the designation of the equilibrium constant by the subscript 50.

$$Y = \frac{\text{number of binding sites occupied}}{\text{total number of binding sites in solution}} \qquad (3.4)$$

$$Y = \frac{[MbO_2]}{[Mb] + [MbO_2]} \qquad (3.5)$$

$$Y = \frac{pO_2}{P_{50} + pO_2} \qquad (3.6)$$

A plot of Eq. 3.6 of Y versus pO_2 generates an oxygen-saturation curve for myoglobin in the form of a rectangular hyperbola (Figure 3.35).

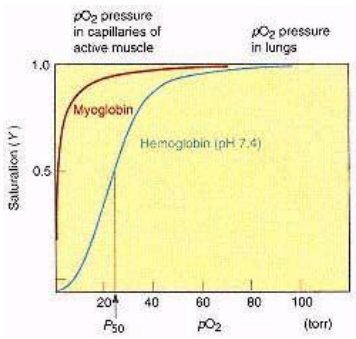


Figure 3.35
Oxygen-binding curves for myoglobin and hemoglobin.

A simple algebraic manipulation of Eq. 3.6 leads to Eq. 3.7. Taking the logarithm of both sides of Eq. 3.7 results in Eq. 3.8, the Hill equation. A plot of log ([Y/(1 – Y)] versus log pO_2, according to Eq. 3.8, yields a straight line with a slope equal to 1 for myoglobin (Figure 3.36). This is the Hill plot, and the slope (n_H) is the Hill coefficient (see Eq. 3.9).

$$\frac{Y}{1-Y} = \frac{pO_2}{P_{50}} \qquad (3.7)$$

$$\log \frac{Y}{1-Y} = \log pO_2 - \log P_{50} \qquad (3.8)$$

Binding of O_2 to Hemoglobin Involves Cooperativity between the Hemoglobin Subunits

Whereas myoglobin has a single O_2-binding site per molecule, hemoglobins, with four monomeric subunits, have four heme-binding sites for O_2. Binding of the four O_2 molecules in hemoglobin is found to be **positively cooperative,** so that the binding of the first O_2 to deoxyhemoglobin facilitates the binding of O_2 to the other subunits in the molecule. Conversely, dissociation of the first O_2 from fully oxygenated hemoglobin, $Hb(O_2)_4$, will make the dissociation of O_2 from the other subunits of the tetramer easier.

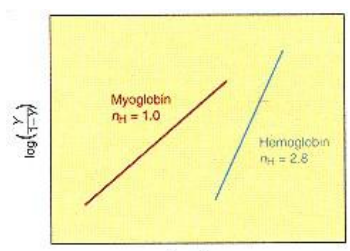


Figure 3.36
Hill plots for myoglobin and hemoglobin HbA_1.

Because of cooperativity in oxygen association and dissociation, the oxygen saturation curve for hemoglobin differs from that for myoglobin. A plot of Y versus pO_2 for hemoglobin is a sigmoidal line, indicating cooperativity in oxygen association (Figure 3.35). A plot of the Hill equation (Eq. 3.9) gives a value of the slope (n_H) equal to 2.8 (Figure 3.36).

$$\log \frac{Y}{1-Y} = n_H \log pO_2 - \text{constant} \qquad (3.9)$$

The meaning of the Hill coefficient to cooperative O_2 association can be evaluated quantitatively as presented in Table 3.10. A parameter known as the cooperativity index, R_x, is calculated, which shows the ratio of pO_2 required to change Y from a value of Y = 0.1 (10% of sites filled) to a value of Y = 0.9 (90% of sites filled) for designated Hill coefficient values found experimentally. For myoglobin, $n_H = 1$, and an 81-fold change in oxygen concentration is required to change from Y = 0.1 to Y = 0.9. For hemoglobin, where positive cooperativity is observed, $n_H = 2.8$ and only a 4.8-fold change in oxygen concentration is required to change the fractional saturation from 0.1 to 0.9.

The Molecular Mechanism of Cooperativity in O_2 Binding

X-ray diffraction data on deoxyhemoglobin show that the ferrous atoms actually sit out of the plane of their porphyrins by about 0.4–0.6 Å. This is thought to occur because of two factors. The electronic configuration of the five-coordinated ferrous atom in deoxyhemoglobin has a slightly larger radius than the distance from the center of the porphyrin to each of the pyrrole nitrogen atoms.

TABLE 3.10 Relationship Between Hill Coefficient (n_H) and Cooperativity Index (R_x)

n_H	R_x		*Observation*
0.5	6560	}	Negative substrate cooperativity
0.6	1520		
0.7	533		
0.8	243		
0.9	132		
1.0	81.0		Noncooperativity
1.5	18.7	}	Positive substrate cooperativity
2.0	9.0		
2.8	4.8		
3.5	3.5		
6.0	2.1		
10.0	1.6		
20.0	1.3		

Source: Based on Table 7.1 in Cornish-Bowden, A. *Principles of Enzyme Kinetics*. London: Butterworths Scientific Publishers, 1976.

Accordingly, the iron can be placed in the center of the porphyrin only with some distortion of the porphyrin conformation. Probably a more important consideration is that if the iron atom sits in the plane of the porphyrin, the proximal His F8 imidazole will interact unfavorably with atoms of the porphyrin. The strength of this unfavorable steric interaction is due, in part, to conformational constraints on the His F8 and the porphyrin in the deoxyhemoglobin conformation that forces the approach of the His F8 toward the porphyrin to a particular path (Figure 3.37). These constraints become less significant in the oxy conformation of hemoglobin.

The conformation with the iron atom out of the plane of the porphyrin is unstrained and energetically favored for the five-coordinate ferrous atom. When O_2 binds the sixth coordinate position of the iron, however, this conformation becomes strained. A more energetically favorable conformation for the O_2 liganded iron is one in which the iron atom is within the plane of the porphyrin structure.

On binding of O_2 to a ferrous atom the favorable free energy of bond formation overcomes the repulsive interaction between His F8 and porphyrin, and the ferrous atom moves into the plane of the porphyrin ring. This is the most thermodynamically stable position for the now six-bonded iron atom; one axial ligand is on either side of the plane of the porphyrin ring, and the steric repulsion of one of the axial ligands with the porphyrin is balanced by the repulsion of the second axial ligand on the opposite side when the ferrous atom is in the center. If the iron atom is displaced from the center, the steric interactions of the two axial ligands with the porphyrin in the deoxy conformation are unbalanced, and the stability of the unbalanced structure will be lower than that of the equidistant conformation. Also, the radius of the iron atom with six ligands is reduced so that it can just fit into the center of the porphyrin without distortion of the porphyrin conformation.

Since steric repulsion between porphyrin and His F8 in the deoxy conformation must be overcome on O_2 association, binding of the first O_2 is characterized by a relatively low affinity constant. However, when O_2 association occurs to the first heme in deoxyhemoglobin, the change in position of the iron atom from above the plane of the porphyrin into the center of the porphyrin triggers

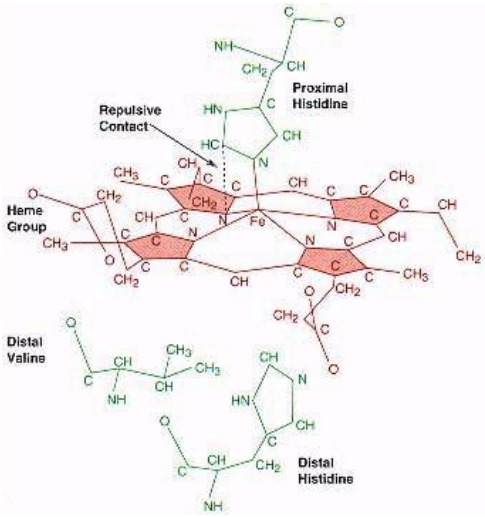


Figure 3.37
Steric hindrance between proximal histidine and porphyrin in deoxyhemoglobin.
From Perutz, M. *Sci Am.*, 239:92, 1978

a conformational change in the whole molecule. The change in conformation results in a greater affinity of O_2 to the other heme sites after the first O_2 has bound.

The conformation of deoxyhemoglobin is stabilized by noncovalent interactions of the quaternary structure at the interface between α and β subunits in which the FG corner of one subunit noncovalently binds to the C helix of the adjacent subunit (Figure 3.38). In addition, ionic interactions stabilize the deoxy

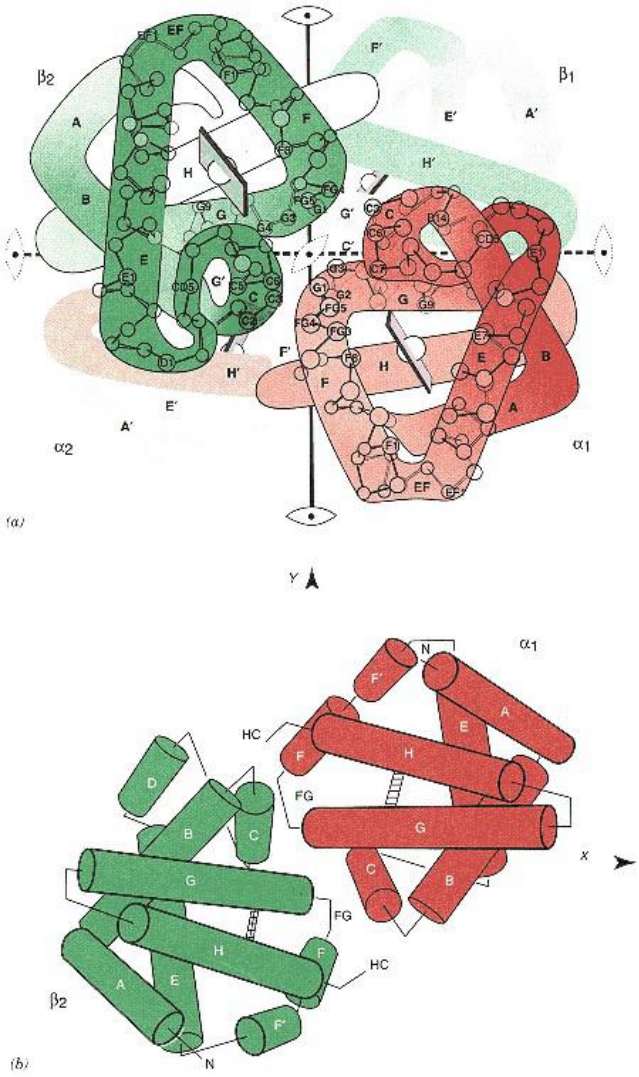


Figure 3.38
Quaternary structure of hemoglobin.
(a) $_1$ $_2$ interface contacts between FG corners and C helix are shown.
(b) Cylinder
representation of $_1$ and $_2$ subunits in hemoglobin molecule showing $_1$ and $_2$ interface contacts between FG corner and C helix, viewed from opposite side of *x–y* plane from (a).
(a) Reprinted with permission from Dickerson, R. E., and Geis, I. *The Structure and Action of Proteins*. Menlo Park, CA: Benjamin, Inc., 1969, p. 56.
(b) Reprinted with permission from Baldwin, J., and Chothia, C. *J. Mol. Biol.* 129:175, 1979.

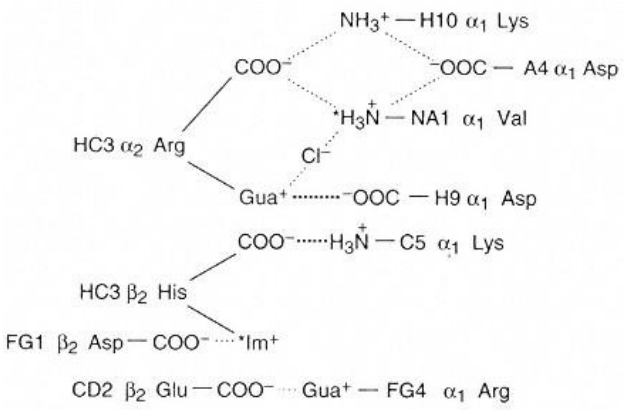


Figure 3.39
Salt bridges between subunits in deoxyhemoglobin.
Im^+ is imidazolium; Gua^+ is guanidinium; starred residues account for approximately 60% of alkaline Bohr effect.
Redrawn from Perutz, M. *Br. Med. Bull.* 32:195, 1976.

conformation of the protein (Figure 3.39). These interactions of the deoxy conformation are now destabilized on the binding of O_2 to one of the heme subunits of a deoxyhemoglobin molecule. The binding of O_2 pulls the Fe^{2+} atom into the porphyrin plane and moves the His F8 toward the porphyrin and with it the F helix of which the His F8 is a part. Movement of the F helix, in turn, moves the FG corner of its subunit, destabilizing the FG noncovalent interaction with the C helix of the adjacent subunit at an $\alpha_1\beta_2$ or $\alpha_2\beta_1$ subunit interface (Figures 3.38 and 3.40).

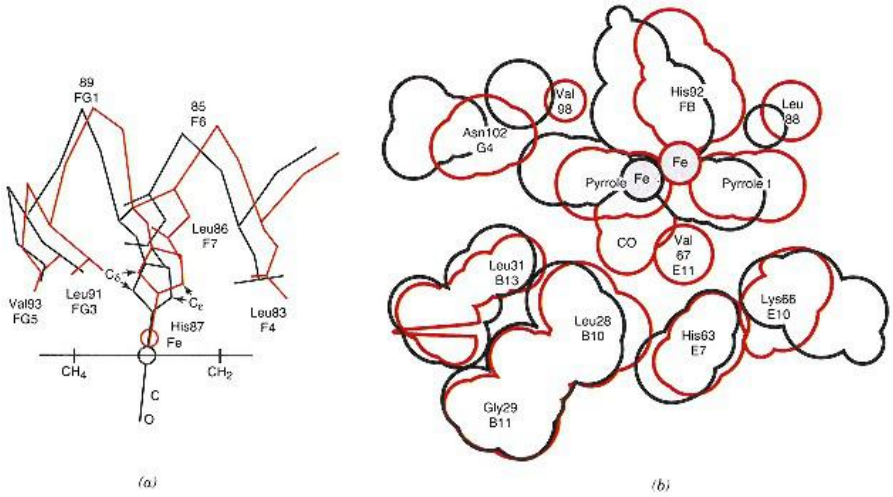


Figure 3.40
Stick and space-filling diagrams drawn by computer graphics showing movements of residues in heme environment on transition from deoxyhemoglobin to oxyhemoglobin.
(a) Black line outlines position of polypeptide chain and His F8 in carbon monoxide hemoglobin, a model for oxyhemoglobin. Red line outlines the same for deoxyhemoglobin. Position of iron atom shown by circle. Movements are for an subunit.
(b) Similar movements in a subunit using space-filling diagram shown. Residue labels centered in density for the deoxyconformation.
Redrawn with permission from Baldwin, J., and Chothia, C. *J. Mol. Biol.* 129:175, 1979.

The FG to C intersubunit contacts act as a "switch," because they exist in two different arrangements with different modes of contact between the FG corner of one subunit and the C helix of the adjacent subunit. The switch in noncovalent interactions between the two positions involves a relative movement of FG and C in adjacent subunits of about 6 Å. In the second position of the "switch," the tertiary conformation of the subunits participating in the FG to C intersubunit contact is less constrained and the adjacent subunit changes to a new tertiary conformation (oxy conformation) even without O_2 bound. This oxy conformation allows the His F8 residues to approach their porphyrins on O_2 association with a less significant steric repulsion than in the deoxy conformation (Figure 3.40). Thus an O_2 molecule binds to the empty hemes in the less constrained oxy conformation more easily than to a subunit conformation held by the quaternary interactions in the deoxy conformation.

In addition, Val E11 in the deoxy conformation of β subunits is at the entrance to the O_2-binding site, where it sterically impedes O_2 association to heme (see Figure 3.33). In the oxy conformation the heme in β subunits moves approximately 1.5 Å further into the heme-binding site, changing the geometric relationship of the O_2-binding site to the Val El1 side chain, so that the Val El1 no longer sterically interferes with O_2 binding. This is an important additional factor that increases affinity of O_2 for the oxy conformation of the β chain over that for the deoxy conformation.

The deoxy conformation of hemoglobin is referred to as the "tense" or **T conformational state.** The oxyhemoglobin conformational form is referred to as the "relaxed" or **R conformational state.** The allosteric mechanism shows how initial binding of the oxygen to one of the heme subunits of the tetrameric molecule pushes the molecular conformation from the T to R conformational state. The affinity constant of O_2 is greater for the R state hemes than the T state by a factor of 150–300, depending on the solution conditions.

The Bohr Effect Involves Dissociation of a Proton on Binding of Oxygen

The equilibrium expression for oxygen association to hemoglobin includes a term that indicates participation of H^+ in the equilibrium.

$$\underset{T}{Hb} + 4O_2 \rightleftharpoons \underset{R}{Hb(O_2)_4} + xH^+ \qquad (3.10)$$

Equation 3.10 shows that the R form is more acidic, and the H^+ dissociate when hemoglobin is changed to the R form. The equivalents of H^+ that dissociate per mole of hemoglobin depends on the pH of the solution and the concentration of other factors that can bind to hemoglobin, such as Cl^- and bisphosphoglycerate (see Chapter 25). At pH 7.4, the value of x may vary from 1.8 to 2.8, depending on the solution conditions. This production of H^+ at an alkaline pH (pH > 6), when deoxyhemoglobin is transformed to oxyhemoglobin, is known as the alkaline **Bohr effect.**

The H^+ are derived from the partial dissociation of acid residues with pK'_a of histidine at blood pH results in conversion of some of its acid form to its conjugate base (imidazole) form, with dissociation of H^+ that forms a part of the Bohr effect. Breakage of

this ion pair with release of protons accounts for 50% of the H^+ released on conversion to the R conformation. Other acid groups in the protein contribute the additional H^+ due to analogous decreases in their pK'_a values on changing from the T to R conformation.

The equilibrium involving hydrogen ions produced by the Bohr effect has important physiological consequences. Cells metabolizing at high rates, with high requirements for molecular oxygen, produce carbonic acid and lactic acid, which act to increase the hydrogen ion concentration in the cell's environment. As the increase in hydrogen ion concentration forces the equilibrium of Eq. 3.10 to the left, from the higher O_2 affinity conformation (R) to the lower affinity conformation (T), an increased amount of oxygen is dissociated from the hemoglobin molecule.

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Questions

J. Baggott and C. N. Angstadt

1. Haptens:

   A. can function as antigens.

   B. strongly bind to antibodies specific for them.

   C. may be macromolecules.

   D. never act as antigenic determinants.

   E. can directly elicit the production of specific antibodies.

2. IgG:

   A. is found primarily in mucosal secretions.

   B. is one of the less common immunoglobulin types.

   C. has the highest molecular weight of all the immunoglobulins.

   D. contains carbohydrate covalently attached to the H chain.

   E. plays an important role in allergic responses.

3. In the three-dimensional structure of immunoglobulins:

   A. β-sheets align edge to edge.

   B. in each chain (H and L) the C and V regions fold onto one another, forming C–V associations.

   C. C_L–V_L associations form the complementary sites for binding antigens.

   D. free–SH groups are preserved to function in tight binding of antigens.

   E. hinge domains connect globular domains

4. Study of the papain hydrolysis products of an antibody indicates:

   A. antibodies are bivalent.

   B. the products have decreased affinity for antigens.

   C. each antibody molecule is hydrolyzed into many small peptides.

   D. the hypervariable sequences are in the hinge region of the intact molecule.

   E. none of the above is true.

5. In immunoglobulins all of the following are true EXCEPT:

   A. there are four polypeptide chains.

   B. there are two copies of each type of chain.

   C. all chains are linked by disulfide bonds.

   D. carbohydrate is covalently bound to the protein.

   E. immunoglobulin class is determined by the C_L regions.

6. Serine proteases:

   A. hydrolyze peptide bonds involving the carboxyl groups of serine residues.

   B. are characterized by having several active sites per molecule, each containing a serine residue.

   C. are inactivated by reacting with one molecule of diisopropyl-fluorophosphate per molecule of protein.

   D. are exopeptidases.

   E. are synthesized in an active form in eukaryotes.

7. The active sites of all serine proteases contain which of the following amino acid residues?

   A. asparagine

   B. γ-carboxyglutamate

   C. histidine

   D. lysine or arginine

   E. threonine

8. All of the following are characteristic of serine proteases as a class EXCEPT:

   A. only one serine residue is catalytically active.

   B. natural protein substrates and inhibitors bind very tightly to the protease.

   C. the genes that code for them are analogously organized.

   D. catalytic units exhibit two structural domains of dramatically different size.

   E. in eukaryotes, the serine proteases are produced initially as zymogens.

9. All of the following serine proteases exhibit sequence homology EXCEPT:

   A. chymotrypsin.

   B. elastase.

   C. *Streptomyces griseus* protease.

   D. subtilisin.

   E. trypsin.

Refer to the following for Questions 10–13.

   A. helix–turn–helix

   B. leucine zipper

   C. zinc finger

   D. all of the above

   E. none of the above

10. Not a DNA-binding motif.

11. Contains a single α-helix.

12. Two domains form a β-pleated sheet, which fits over the DNA double helix.

13. Found in proteins which bind to DNA.

14. The α-chain appears in all normal human hemoglobins EXCEPT:

   A. HbA_1.

   B. HbA_2.

   C. HbF.

   D. Hb Gower-1.

15. Hemoglobin and myoglobin both have all of the following characteristics EXCEPT:

   A. consist of subunits designed to provide hydrogen bonds to and nonpolar interaction with other subunits.

   B. highly α-helical.

   C. bind one molecule of heme per globin chain.

   D. bind heme in a hydrophobic pocket.

   E. can bind one O_2 per heme.

16. Hemoglobin, but not myoglobin, when it binds oxygen, exhibits:

A. a hyperbolic saturation curve.

B. a Hill coefficient of 1.

C. positive cooperativity.

D. a cooperativity index of 81.

17. All of the following are believed to contribute to the stability of the deoxy or T conformation of hemoglobin EXCEPT:

A. the larger ionic radius of the six-coordinated ferrous ion as compared to the five-coordinated ion.

B. steric interaction of His F8 with the porphyrin ring.

C. interactions between the FG corner of one subunit and the C helix of the adjacent subunit.

D. a valyl residue that tends to block O_2 from approaching the hemes of the β-chains.

18. In the Bohr effect:

A. oxygen is released with increasing difficulty as the pH decreases.

B. the R form of hemoglobin is more acidic than the T form.

C. histidine 146 (β) interacts with a nearby Cl^-.

D. cells with higher voluntary oxygen demand are deprived to ensure adequate oxygen for other tissues.

E. bisphosphoglycerate decreases the oxygen affinity of hemoglobin.

Answers

1. B Haptens are small molecules and cannot alone elicit antibody production; thus they are not antigens. They can act as antigenic determinants if covalently bound to a larger molecule, and free haptens may bind strongly to the antibodies thereby produced (p. 88).

2. D All immunoglobulins are glycoproteins. A: IgA is associated with mucosal secretions. B: IgG is the most common immunoglobulin type. C: IgM has the highest molecular weight (Table 3.1, p. 90) E: IgE plays an important role in allergic responses (Clin. Corr. 3.2, p. 92).

3. E See Figures 3.4–3.7, p. 93. A: The β-sheets align face-to-face. D: Antigen binding is noncovalent.

4. A In these hydrolysis experiments, three fragments are produced: two identical Fab fragments, each of which binds antigen with an affinity similar to that of the whole antibody molecule, and one Fc fragment, which does not bind antigens (p. 92).

5. E There are two copies of each of two types of polypeptide chain (p. 89).

6. C This is the distinguishing characteristic of the serine proteases, and of the serine hydrolases in general. A: They have various specificities (p. 99). B: There is only one active site per molecule (p. 103). D: They are all endopeptidases (p. 98). E: In eukaryotes they are synthesized as inactive precursors, zymogens, or proenzymes (p. 105).

7. C (p. 103). A: Aspartate, not asparagine, is involved. B: γ-Carboxyglutamate is essential to some of the serine proteases, but it is not at the active site. D: These are the substrate specificities of the trypsin-like proteases.

8. D The domains are of about equal size (p. 105).

9. D The bacterial protease subtilisin may be an example of converging evolution (p. 106). See also Table 3.7, p. 107.

10. B The leucine zipper binds two subunits in a head-to-head manner but does not itself interact with DNA (p. 110).

11. C See p. 108 and Figure 3.23.

12. E This describes the TATA box-binding protein (p. 112).

13. D

14. D Hb Gower-1 has the structure $\zeta_2\varepsilon_2$ (p. 114, Table 3.8).

15. A Hemoglobin has four chains and four oxygen-binding sites, whereas myoglobin has one chain and one oxygen-binding site. Each oxygen-binding site is a heme (p. 115).

16. C Hemoglobin's Hill coefficient of 2.8 indicates positive cooperativity (p. 119). A: See p. 118, Figure 3.35. B: Myoglobin has a Hill coefficient of 1. D: A cooperativity index of 81 indicates noncooperativity; hemoglobin's lower value of 4.8 reflects cooperative oxygen binding (p. 119).

17. A Six-coordinated ferrous ion has a smaller ionic radius than the five-coordinated species and just fits into the center of the porphyrin ring without distortion (p. 120).

18. B His 146 (β) is a major contributor to the Bohr effect. Thus its pK'_a will be lower (it will be a stronger acid) in oxyhemoglobin (p. 123). E: This is true but is unrelated to the Bohr effect.

Chapter 4—
Enzymes:
Classification, Kinetics, and Control

J. Lyndal York

4.1— General Concepts

Enzymes are specialized proteins that function in the acceleration of chemical reactions. Many reactions required for normal activity of cells would not proceed fast enough at the pH and temperature of the body without these specialized proteins. The term defining the speed of a chemical reaction, whether catalyzed or uncatalyzed, is **rate** or **velocity.** Rate (velocity) is the change in amount (moles, grams) of starting materials or products per unit time. Enzymes increase the rate by acting as catalysts. A **catalyst** increases the rate of a chemical reaction but is not itself changed in the process. An enzyme may become temporarily covalently bound to a molecule being transformed during intermediate stages of the reaction but at the end of the reaction the enzyme will again be in its original form as the product is released.

Two important characteristics of enzyme catalysts are that the enzyme is not changed as a result of catalysis and the enzyme does not change the equilibrium constant of the reaction but simply increases the rate at which the reaction approaches equilibrium. As will be discussed later, it accomplishes the rate increase by lowering the barrier to reaction; that is, it lowers the energy of activation. Therefore a catalyst increases the rate but does not change the thermodynamic properties of the system with which it is interacting.

Several terms need to be defined before we enter into a discussion of the mechanism of enzyme action. An **apoenzyme** is the protein part of an enzyme without any cofactors or prosthetic groups that may be required for the enzyme to be functional. The apoenzyme is catalytically inactive. Not all enzymes require cofactors or prosthetic groups. **Cofactors** are small organic or inorganic molecules that an apoenzyme requires for its activity. For example, in lysine oxidase copper is loosely bound but is required for the enzyme to be active. A **prosthetic** group is similar to a cofactor but is tightly bound to an apoenzyme. For example, in the cytochromes, the heme prosthetic group is very tightly bound and requires strong acids to disassociate it from the apocytochrome. Addition of a cofactor or prosthetic group to the apoprotein yields the **holoenzyme,** that is, the active enzyme. The molecule acted upon by the enzyme to form product is the **substrate.** Since most reactions are reversible, the products of the forward reaction become substrates for the reverse reaction.

Enzymes have a great deal of specificity. For example, glucose oxidase will oxidize glucose but not galactose. The specificity resides in a particular region on the enzyme surface, the **substrate-binding site,** a particular arrangement of amino acid side chains in the polypeptide that is specially formulated to

bind a specific substrate. Some enzymes have broad specificity; glucose, mannose, and fructose are phosphorylated by hexokinase, whereas glucokinase is specific for glucose. The substrate-binding site may contain the **active site.** In some cases, however, the active site may not be within the substrate-binding site but may be contiguous to it in the primary sequence. In other instances the active-site residues lie in distant regions of the primary sequence but are brought adjacent to the substrate-binding site by folding in the tertiary structure. The **active site** contains the machinery, in the form of particular amino acid side chains, involved in catalyzing the reaction.

Some enzymes have variants called **isoenzymes** (isozymes) that catalyze the same chemical reaction. Isoenzymes are electrophoretically distinguishable because of mutations in one or more amino acids in noncritical areas of the protein.

Some enzymes have a region of the molecule, the **allosteric** site, that is not at the active site or substrate-binding site but is a unique site where small molecules bind and effect a change in the substrate-binding site or the activity occurring in the active site. The binding of a specific small molecule at the allosteric site causes a change in the conformation of the enzyme. This can cause the active site to become either more active or less active by increasing or decreasing the affinity of the binding site for substrate. Such interactions regulate the enzyme's activity and are discussed in detail on page 151.

4.2— Classification of Enzymes

The International Union of Biochemistry and Molecular Biology (IUBMB) has established a system whereby all enzymes are classified into six major classes, each subdivided into subclasses that are further subdivided. In naming an enzyme, the substrates are stated first, followed by the reaction type to which the ending -ase is affixed. For example, alcohol dehydrogenase is alcohol:NAD^+ oxidoreductase because it catalyzes an oxidation–reduction reaction and the electron donor is an alcohol and the acceptor is NAD^+. Many common names persist but are not very informative. For example, "aldolase" does not tell much about the substrates, although it does identify the reaction type. We will use trivial names recognized by the IUBMB and that are in common usage. Table 4.1 summarizes the six major classes and subclasses of enzymes.

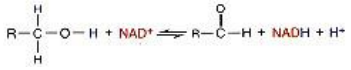


Figure 4.1
Oxidation of ethanol by alcohol dehydrogenase.

Class 1— Oxidoreductases

These enzymes catalyze **oxidation–reduction reactions.** For example, alcohol:NAD^+ oxidoreductase (alcohol **dehydrogenase**) catalyzes the oxidation of an alcohol to an aldehyde. It removes two electrons and two hydrogen atoms from the alcohol to yield an aldehyde, and, in the process, the two electrons originally in the carbon–hydrogen bond of the alcohol are transferred to the NAD^+, which is reduced (Figure 4.1). NAD^+, whose structure is presented in Figure 4.19, is a cofactor that mediates many biological oxidation–reduction reactions. The redox site in NAD^+ is shown in Figure 4.20. In addition to the alcohol and aldehyde functional groups, dehydrogenases also act on the following functional groups as electron donors: $–CH_2–CH_2–$, $–CH_2–NH_2$, and –CH=NH, as well as the cofactors NADH and NADPH.

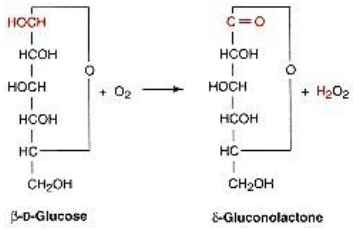


Figure 4.2
Oxidation of glucose by glucose oxidase.

There are other subclasses of the oxidoreductases. **Oxidases** transfer two electrons from the donor to oxygen, resulting usually in hydrogen peroxide (H_2O_2) formation. For example, **glucose oxidase** catalyzes the reaction shown in Figure 4.2. **Cytochrome oxidase** produces H_2O rather than H_2O_2. **Oxygenases** catalyze the incorporation of oxygen into a substrate. With dioxygenases both atoms of O_2 are incorporated in a single product, whereas with the monooxygenases a single oxygen atom is incorporated as a hydroxyl group

TABLE 4.1 Summary of the Enzyme Classes and Major Subclasses

1. Oxidoreductases Dehydrogenases Oxidases Reductases Peroxidases Catalase Oxygenases Hydroxylases	2. Transferases Transaldolase and transketolase Acyl, methyl, glucosyl, and phosphoryltransferase Kinases Phosphomutases
3. Hydrolases Esterases Glycosidases Peptidases Phosphatases Thiolases Phospholipases Amidases Deaminases Ribonucleases	4.Lyases Decarboxylases Aldolases Hydratases Dehydratases Synthases Lyases
5. Isomerases Racemases Epimerases Isomerases Mutases (not all)	6. Ligases Synthetases Carboxylases

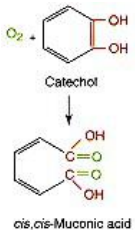


Figure 4.3
Oxygenation of catechol by an oxygenase.

and the other oxygen atom is reduced to water by electrons from the substrate or from a second substrate that is not oxygenated. **Catechol oxygenase** catalyzes the dioxygenase reaction (Figure 4.3); steroid hydroxylase illustrates a monooxygenase (mixed function oxygenase) reaction (Figure 4.4). **Peroxidases** utilize H_2O_2 rather than oxygen as the oxidant. NADH peroxidase catalyzes the reaction

$$NADH + H^+ + H_2O_2 \leftrightharpoons NAD^+ + 2H_2O$$

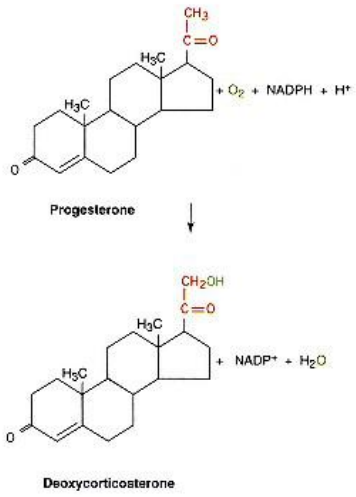


Figure 4.4
Hydroxylation of progesterone by a monooxygenase.

Catalase is unique in that H_2O_2 serves as both donor and acceptor. Catalase functions in the cell to detoxify H_2O_2:

$$H_2O_2 + H_2O_2 \rightleftharpoons O_2 + 2H_2O$$

Class 2— Transferases

These enzymes transfer functional groups between donors and acceptors. The amino, acyl, phosphate, one-carbon, and glycosyl groups are the major moieties that are transferred. **Aminotransferases (transaminases)** transfer an amino group from one amino acid to an α-keto acid acceptor, resulting in the formation of a new amino acid and a new keto acid (Figure 4.5). **Kinases** are the phosphorylating enzymes that catalyze the transfer of the γ phosphoryl group from ATP or another nucleoside triphosphate to alcohol or amino group acceptors. For example, glucokinase catalyzes the phosphorylation of glucose (Figure 4.6).

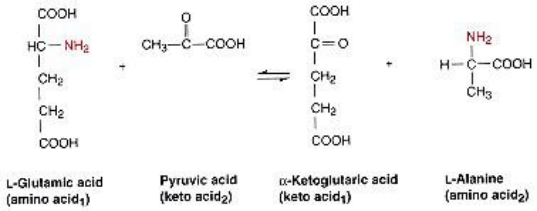


Figure 4.5
Examples of a reaction catalyzed by an aminotransferase.

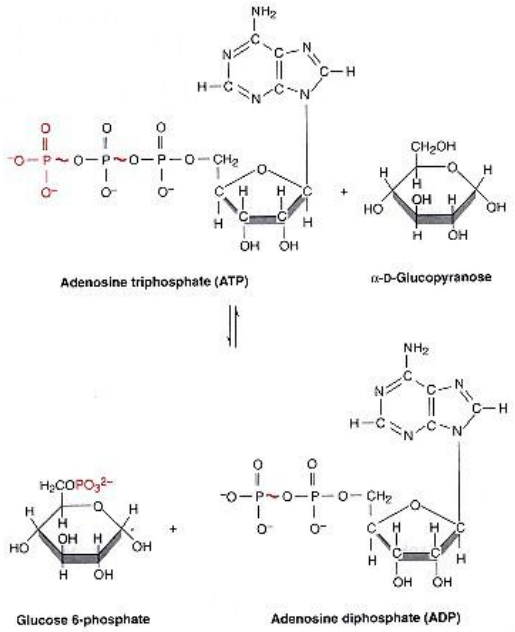


Figure 4.6
Phosphorylation of glucose by a kinase.

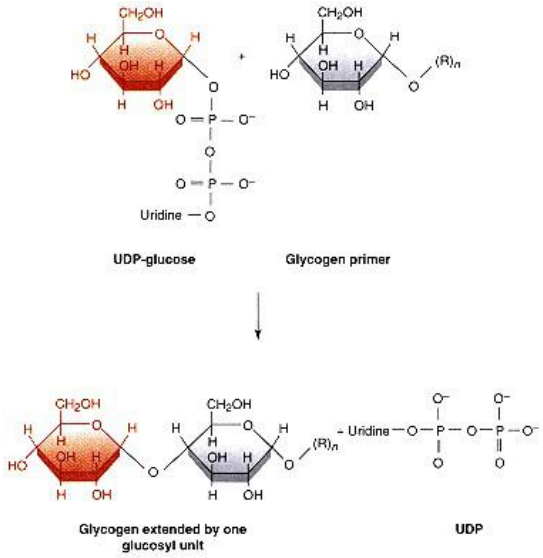


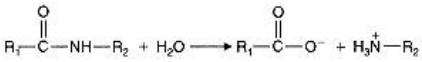
Figure 4.7
A transferase reaction—synthesis of glycogen.

Glycogen synthesis depends on glucosyltransferases, which transfer an activated glucosyl residue to a glycogen primer. The phosphoester bond in uridine diphosphoglucose is labile, which allows the glucose to be transferred to the growing end of the glycogen primer as indicated in Figure 4.7.

Although a polymer is synthesized, the reaction is not of the ligase type reaction; see Class 6.

Class 3—
Hydrolases

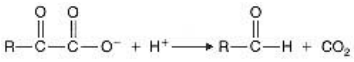
This group of enzymes can be considered as a special class of the transferases in which the donor group is transferred to water. The generalized reaction involves the hydrolytic cleavage of C–O, C–N, O–P, and C–S bonds. The cleavage of a peptide bond is a good example of this reaction:



Proteolytic enzymes are a special class of hydrolases called **peptidases.**

Class 4—
Lyases

Lyases add or remove the elements of water, ammonia, or carbon dioxide. **Decarboxylases** remove the element of CO_2 from α- or β-keto acids or amino acids:



Dehydratases remove H_2O in a dehydration reaction. Fumarase converts fumarate to malate (Figure 4.8).

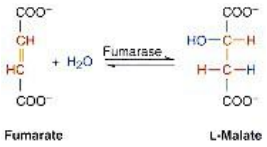


Figure 4.8
The fumarase reaction.

Class 5— Isomerases

This very heterogeneous group of enzymes catalyze isomerizations of several types. These include cis–trans and aldose–ketose interconversions. Isomerases that catalyze inversion at asymmetric carbon atoms are either **epimerases** or **racemases** (Figure 4.9). **Mutases** involve intramolecular transfer of a group such as a phosphoryl. The transfer may be direct but can involve a phosphorylated enzyme as an intermediate. Phosphoglycerate mutase catalyzes conversion of 2-phosphoglycerate to 3-phosphoglycerate (Figure 4.10).

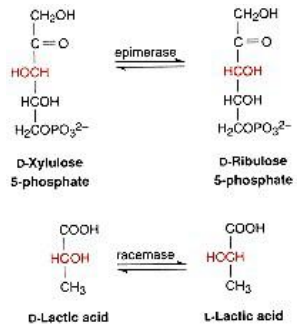


Figure 4.9
Examples of reactions catalyzed by an epimerase and a racemase.

Class 6— Ligases

Since to ligate means to bind, these enzymes are involved in synthetic reactions where two molecules are joined at the expense of a "high-energy phosphate bond" of ATP. The term **synthetase** is reserved for this particular group of enzymes. The formation of aminoacyl tRNAs, acyl coenzyme A, and glutamine and the addition of CO_2 to pyruvate are reactions catalyzed by ligases. Pyruvate carboxylase is a good example of a ligase enzyme (Figure 4.11). The substrates bicarbonate and pyruvate are ligated to form a four-carbon (C4) α-keto acid.

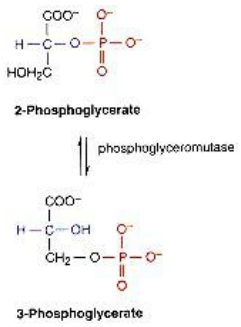


Figure 4.10
Interconversion of the 2- and 3-phosphoglycerates.

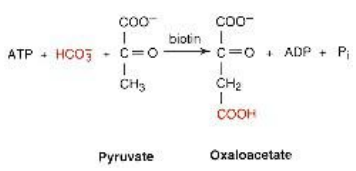


Figure 4.11
Pyruvate carboxylase reaction.

4.3— Kinetics

Kinetics Studies the Rate of Change of Reactants to Products

Since enzymes affect the rate of chemical reactions, it is important to understand basic chemical kinetics and how kinetic principles apply to enzyme-catalyzed reactions. **Kinetics** is a study of the rate of change of reactants to products. **Velocity** is expressed in terms of change in the *concentration* of substrate or product per unit time, whereas **rate** refers to changes in *total quantity* (moles or grams) per unit time. Biochemists tend to use these terms interchangeably.

The velocity of a reaction A P is determined from a progress curve or velocity profile of a reaction. The progress curve can be determined by following the disappearance of reactants or the appearance of product at several different times. In Figure 4.12, product appearance is plotted against time. The slope of tangents to the progress curve yields the instantaneous velocity at that point in time. The initial velocity is an important parameter in the assay of enzyme concentration. Note that the velocity changes constantly as the reaction proceeds to equilibrium, where it becomes zero. Mathematically, the velocity is expressed as

$$\text{Velocity} = v = \frac{-d[\text{A}]}{dt} = \frac{d[\text{P}]}{dt} \qquad (4.1)$$

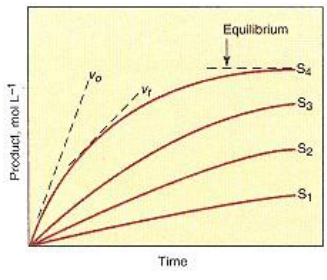


Figure 4.12
Progress curves for an enzyme- catalyzed reaction. The initial velocity (v_0) of the reaction is determined from the slope of the progress curve at the beginning of the reaction. The initial velocity increases with increasing substrate concentration (S_1–S_4) but reaches a limiting value characteristic of each enzyme. The velocity at any time, t, is denoted as v_t.

and represents the change in concentration of reactants or products per unit time.

The Rate Equation

Determination of the velocity of a reaction reveals nothing about the stoichiometry of the reactants and products or about the reaction mechanism. An equation is needed that relates the experimentally determined initial velocity to the concentration of reactants. This is the velocity or rate equation. In the reaction A P, the velocity equation is

$$\frac{-d[A]}{dt} = v = k[A]^n \qquad (4.2)$$

Thus the observed initial velocity depends on the starting concentration of A to the *n*th power multiplied by a proportionality constant (***k***). The latter is known as the **rate constant**. The exponent *n* is usually an integer from 1 to 3 that is required to satisfy the mathematical identity of the velocity expression.

Characterization of Reactions Based on Order

Another term useful in describing a reaction is the **order of reaction.** Empirically the order is determined as the sum of the exponents on each concentration term in the rate expression. In the case under discussion the reaction is **first order,** since the velocity depends on the concentration of A to the first power, $v = k[A]^1$. In the reaction A + B C, if the order with respect to A and B is 1, that is, $v = k[A]^1[B]^1$, overall the reaction is second order. Note that the order of reaction is independent of the stoichiometry of the reaction; that is, if the reaction were third order, the rate expression could be either $v = k[A][B]^2$ or $v = [A]^2[B]$, depending on the order in A and B. Since the velocity of the reaction is constantly changing as the reactant concentration changes, first-order reaction conditions would not be ideal for assaying an enzyme-catalyzed reaction because one would have two variables, the changing substrate concentration and the unknown enzyme concentration.

If the differential first-order rate expression Eq. 4.2 is integrated, one obtains

$$k_1 \cdot t = 2.3 \log\left(\frac{[A]}{[A] - [P]}\right) \qquad (4.3)$$

where [A] is the initial reactant concentration and [P] is the concentration of product formed at time *t*. The first-order rate constant k_1 has the units of reciprocal time. If the data shown in Figure 4.12 were replotted as log [P] versus time for any one of the substrate concentrations, a straight line would be obtained whose slope is equal to $k_1/2.303$. The rate constant k_1 should not be confused with the rate or velocity of the reaction.

Many biological processes proceed under first-order conditions. The clearance of many drugs from the blood by peripheral tissues is a first-order process. A specialized form of the rate equation can be used in these cases. If we define $t_{1/2}$ as the time required for the concentration of the reactants or the blood level of a drug to be reduced by one-half the initial value, then Eq. 4.3 reduces to

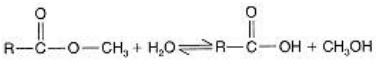
$$k_1 \cdot t_{1/2} = 2.3 \log\left(\frac{1}{1 - \frac{1}{2}}\right) = 2.3 \log 2 = 0.69 \qquad (4.4)$$

or

$$t_{1/2} = \frac{0.69}{k_1} \qquad (4.5)$$

Note that $t_{1/2}$ is not one-half the time required for the reaction to be completed. The term $t_{1/2}$ is referred to as the half-life of the reaction.

Many **second-order** reactions that involve water or any one of the reactants in large excess can be treated as pseudo-first-order reactions. In the hydrolysis of an ester,



the second-order rate expression is

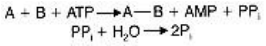
$$\text{velocity} = v = k_2[\text{ester}]^1[H_2O]^1 \qquad (4.6)$$

but since water is in abundance (55.5 M) compared to the ester (10^{-3}–10^{-2} M), the system obeys the first-order rate law Eq. 4.2, and the reaction appears to proceed as if it were a first-order reaction. Reactions in the cell that involve hydration, dehydration, or hydrolysis are pseudo-first-order.

The rate expression for the **zero-order** reaction is $v = k_0$. Note that there is no concentration term for reactants; therefore the addition of more reactant does not augment the rate. The disappearance of reactant or the appearance of product proceeds at a constant velocity irrespective of reactant concentration. The units of the rate constant are concentration per unit time. Zero-order reaction conditions only occur in catalyzed reactions where the concentration of reactants is large enough to saturate all the catalytic sites. Under these conditions the catalyst is operating at maximum velocity, and all catalytic sites are filled; therefore addition of more reactant cannot increase the rate.

Reversibility of Reactions

Although most chemical reactions are reversible, some directionality is imposed on particular steps in a metabolic pathway by rapid removal of end product by subsequent reactions in the pathway. Many ligase reactions involving the nucleoside triphosphates result in release of pyrophosphate (PP_i). These reactions are rendered irreversible by the hydrolysis of the pyrophosphate to 2 moles of inorganic phosphate, P_i. Schematically,



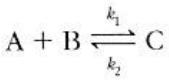
Conversion of the "high-energy" pyrophosphate to inorganic phosphate imposes irreversibility on the system by virtue of the thermodynamic stability of the products.

For reactions that are reversible, the equilibrium constant for

is

$$K_{eq} = \frac{[C]}{[A][B]} \qquad (4.7)$$

and can also be expressed in terms of rate constants of the forward and reverse reactions:



where

$$\frac{k_1}{k_2} = K_{eq} \qquad (4.8)$$

Equation 4.8 shows the relationship between thermodynamic and kinetic quantities. The term K_{eq} is a thermodynamic expression of the state of the system, while k_1 and k_2 are kinetic expressions that are related to the speed at which that state is reached.

Enzymes Show Saturation Kinetics

Terminology

Enzyme activity is usually expressed as micromoles (μ mol) of substrate converted to product per minute under specified assay conditions. One standard unit of enzyme activity (U) is that activity that catalyzes transformation of 1 μ mol min^{-1}. **Specific activity** of an enzyme preparation is defined as the number of enzyme units per milligram of protein (μ mol min^{-1} mg of $protein^{-1}$ or U/mg of protein). This expression, however, does not indicate whether the sample tested contains only the enzyme protein; during enzyme purification the value will increase as contaminating protein is removed. The **catalytic constant,** or **turnover number,** of an enzyme is equal to the units of activity per mole of enzyme (μ mol/min/mol of enzyme). Where the enzyme has more than one catalytic center, the catalytic constant is often given on the basis of the particle weight of the subunit rather than the molecular weight of the entire protein. The Commission on Enzyme Nomenclature of the International Union of Biochemistry and Molecular Biology recommends that enzyme activity be expressed in units of moles per second, instead of micromoles per minute, to conform with the rate constants used in chemical kinetics. A new unit, the **Katal** (abbreviated kat), is proposed where 1 kat denotes conversion of 1 mol substrate per second. Activity can be expressed, however, as millikatals (mkat), microkatals (μ kat), and so forth. The specific activity and catalytic constant can also be expressed in katals.

The catalytic constant or turnover number allows direct comparison of relative catalytic ability between enzymes. For example, the constants for catalase and α-amylase are 5×10^6 and 1.9×10^4, respectively, indicating that catalase is about 2500 times more active than amylase.

Maximum velocity, V_{max}, is the velocity obtained under conditions of substrate saturation of the enzyme under specified conditions of pH, temperature, and ionic strength. V_{max} is a constant for a given enzyme.

Interaction of Enzyme and Substrate

The initial velocity of an enzyme-catalyzed reaction is dependent on the concentration of substrate (S) (Figure 4.12). As concentration of substrate increases (S_1–S_4), initial velocity increases until the enzyme is completely saturated with substrate. If initial velocities obtained at given substrate concentrations are plotted (Figure 4.13), a rectangular hyperbola is obtained like that obtained for binding of oxygen to myoglobin as a function of increasing oxygen pressure. In general, a rectangular hyperbola is obtained for any process that involves interaction or binding of reactants or other substances at a specific but limited number of sites. The velocity of the reaction reaches a maximum at the point at which all the available sites are saturated. The curve in Figure 4.13 is referred

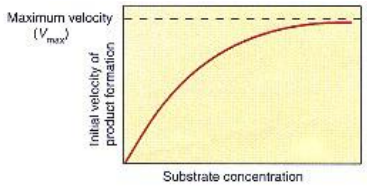


Figure 4.13
Plot of velocity versus substrate for an enzyme-catalyzed reaction.
Initial velocities are plotted against the substrate concentration at which they were determined. The curve is a rectangular hyperbola, which asymptotically approaches the maximum velocity possible with a given amount of enzyme.

to as the substrate saturation curve of an enzyme-catalyzed reaction and reflects the fact that an enzyme has a specific binding site for the substrate. Enzyme (E) and substrate must interact in some way if the substrate is to be converted to products. Initially there is formation of a complex between the enzyme and substrate:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (4.9)$$

The rate constant for formation of the ES complex is defined as k_1, and the rate constant for disassociation of the ES complex is defined as k_2. So far, we have described only an equilibrium binding of enzyme and substrate. The chemical event in which bonds are made or broken occurs in the ES complex. The conversion of substrate to products (P) then occurs from the ES complex with a rate constant k_3. Therefore, Eq. 4.9 is transformed to

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (4.10)$$

Equation 4.10 is a general statement of the mechanism of enzyme action. The equilibrium between E and S can be expressed as an affinity constant, K_a, only if the rate of the chemical phase of the reaction, k_3, is small compared to k_2; then $K_a = k_1/k_2$. Earlier we used K_{eq} to describe chemical reactions. In enzymology the association or affinity constant K_a is preferred.

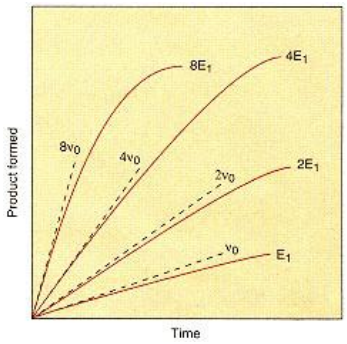


Figure 4.14
Progress curves at variable concentrations of enzyme and saturating levels of substrate.
The initial velocity (v_0) doubles as the enzyme concentration doubles. Since the substrate concentrations are the same, the final equilibrium concentrations of product will be identical in each case; however, equilibrium will be reached at a slower rate in those assays containing small amounts of enzyme.

The **initial velocity**, v_0, of an enzyme-catalyzed reaction is dependent on amount of substrate present and on enzyme concentration. Figure 4.14 shows progress curves for increasing concentrations of enzyme, where there is enough substrate initially to saturate the enzyme at all levels. The initial velocity doubles as the concentration of enzyme doubles. At the lower concentrations of enzyme, equilibrium is reached more slowly than at higher concentrations, but the final equilibrium position is the same.

From this discussion, we can conclude that the velocity of an enzyme reaction is dependent on both substrate and enzyme concentrations.

Formulation of the Michaelis–Menten Equation

In the discussion of chemical kinetics, rate equations were developed so that velocity of the reaction could be expressed in terms of substrate concentration. This approach also holds for enzyme-catalyzed reactions, where the goal is to develop a relationship that will allow the velocity of a reaction to be correlated with the amount of enzyme. First, a rate equation must be developed that relates the velocity of the reaction to the substrate concentration.

Development of this rate equation, known as the **Michaelis–Menten equation,** requires three basic assumptions. The first is that the ES complex is in a **steady state;** that is, during the initial phases of the reaction, the concentration of the ES complex remains constant, even though many molecules of substrate are converted to products via the ES complex. The second assumption is that under saturating conditions all of the enzyme is converted to ES complex, and none is free. This occurs when the substrate concentration is high. The third assumption is that if all the enzyme is in the ES complex, then the rate of formation of products will be maximal; that is,

$$V_{max} = k_3[ES] \qquad (4.11)$$

If we then write the steady-state expression for formation and breakdown of the ES complex as

$$K_m = \frac{k_2 + k_3}{k_1} \qquad (4.12)$$

the rate expression obtained by suitable algebraic manipulation becomes

$$\text{Velocity} = v = \frac{V_{max} \cdot [S]}{K_m + [S]} \qquad \textbf{(4.13)}$$

The complete derivation of this equation is at the end of this section. The two constants in this rate equation, V_{max} and K_m, are unique to each enzyme under specific conditions of pH and temperature. For enzymes in which $k_3 \ll k_2$, K_m becomes the reciprocal of the enzyme–substrate binding constant, and V_{max} reflects the catalytic phase of the enzyme mechanism as suggested by Eq. 4.11. Thus, in this model, activity of the enzyme can be separated into two phases: binding of substrate followed by chemical modification of the substrate. This biphasic nature of the enzyme mechanism is reinforced in the clinical example discussed in Clin. Corr. 4.1.

CLINICAL CORRELATION 4.1

A Case of Gout Demonstrates Two Phases in the Mechanism of Enzyme Action

The two phases of the Michaelis–Menten model of enzyme action, binding followed by modification of substrate, are illustrated by studies on a family with gout. The patient excreted three times the normal amount of uric acid per day and had markedly increased levels of 5 -phosphoribosyl-α-pyrophosphate (PRPP) in his red blood cells. PRPP is an intermediate in the biosynthesis of AMP and GMP, which are converted to ATP and GTP. Uric acid arises directly from degradation of AMP and GMP. Assays *in vitro* revealed that the patient's red cell PRPP synthetase activity was increased threefold. The pH optimum and the K_m of the enzyme for ATP and ribose 5-phosphate were normal, but V_{max} was increased threefold! This increase was not due to an increase in the amount of enzyme; immunologic testing with a specific antibody to the enzyme revealed similar quantities of the enzyme protein as in normal red cells. This finding demonstrates that the binding of substrate as reflected by K_m and the subsequent chemical event in catalysis, which is reflected in V_{max}, are separate phases of the overall catalytic process. This situation holds only for those enzyme mechanisms in which $k_3 \gg k_2$.

Becker, M. A., Kostel, P. J., Meyer, L. J., and Seegmiller, J. E. Human phosphoribosylpyrophosphate synthetase: increased enzyme specific activity in a family with gout and excessive purine synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 70:2749, 1973.

Significance of K_m

The concept of K_m may appear to have no physiological or clinical relevance. The truth is quite the contrary. As discussed in Section 4.9, all valid enzyme assays performed in the clinical laboratory are based on knowledge of K_m values for each substrate. In terms of the physiological control of glucose and phosphate metabolism, two **hexokinases** have evolved, one with a high K_m and one with a low K_m for glucose. Together, they contribute to maintaining steady-state levels of blood glucose and phosphate, as discussed on page 284.

In general, K_m values are near the concentrations of substrate found in cells. Perhaps enzymes have evolved substrate-binding sites with affinities comparable to *in vivo* levels of their substrates. Occasionally, mutation of an enzyme-binding site occurs, or an isoenzyme with an altered K_m is expressed. Either of these events can result in an abnormal physiology. An interesting example (Clin. Corr. 4.2) is the expression of only the atypical form of **aldehyde dehy-drogenase** in people of Asiatic origin.

Note that if one allows the initial velocity, v_0, to be equal to 1/2 V_{max} in Eq. 4.13, K_m will be equal to [S]:

$$\tfrac{1}{2}V_{max} = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

$$K_m + [S] = \frac{2\,V_{max} \cdot [S]}{V_{max}}$$

$$K_m = [S]$$

Thus, from a substrate saturation curve, the numerical value of K_m can be derived by graphical analysis (Figure 4.15). Here the K_m is equal to the substrate concentration that gives one-half the maximum velocity.

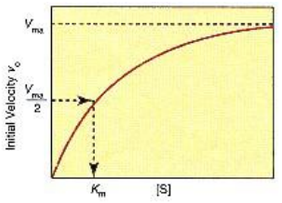


Figure 4.15
Graphic estimation of K_m for the v_0 versus [S] plot.
K_m is the substrate concentration at which the enzyme has half-maximal activity.

Linear Form of the Michaelis–Menten Equation

In practice the determination of K_m from the substrate saturation curve is not very accurate, because V_{max} is approached asymptotically. If one takes the reciprocal of Eq. 4.13 and separates the variables into a format consistent with the equation of a straight line ($y = mx + b$), then

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

A plot of the reciprocal of the initial velocity versus the reciprocal of the initial substrate concentration yields a line whose slope is K_m / V_{max} and whose y-intercept is $1/V_{max}$. Such a plot is shown in Figure 4.16. It is often easier to obtain the K_m from the intercept on the x-axis, which is $-1/K_m$.

This linear form of the Michaelis–Menten equation is often referred to as the **lineweaver–Burk** or **double-reciprocal plot.** Its advantage is that

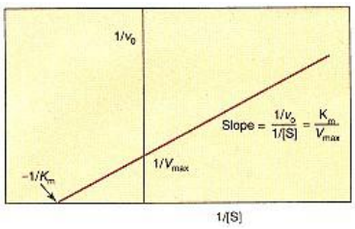


Figure 4.16
Determination of K_m and V_{max} from the Line weaver –Burk double-reciprocal plot.
Plots of the reciprocal of the initial velocity versus the reciprocal of the substrate concentration used to determine the initial velocity yield a line whose x-intercept is $-1/K_m$.

CLINICAL CORRELATION 4.2

The Physiological Effect of Changes in Enzyme K_m Values

The unusual sensitivity of Asians to alcoholic beverages has a biochemical basis. In some Japanese and Chinese, much less ethanol is required to produce vasodilation that results in facial flushing and rapid heart rate than is required to achieve the same effect in Europeans. The physiological effects are due to acetaldehyde generated by liver alcohol dehydrogenase.

$$CH_3CH_2OH + NAD^+ \leftrightharpoons CH_3CHO + H^+ + NADH$$

Acetaldehyde is normally removed by a mitochondrial aldehyde dehydrogenase that converts it to acetate. In some Asians, the normal form of the mitochondrial aldehyde dehydrogenase, with a low K_m for acetaldehyde, is missing. These individuals have only the cytosolic high K_m (lower affinity) form of the enzyme, which leads to a high steady-state level of acetaldehyde in the blood after alcohol consumption. This accounts for the increased sensitivity to alcohol.

Crabb, D. W., Edenberg, H. J., Bosron, W. F., and Li, T.-K. Genotypes for aldehyde dehydrogenase deficiency and alcohol sensitivity: The ALDH22 allele is dominant. *J. Clin. Invest.* 83:314, 1989.

statistically significant values of K_m and V_{max} can be obtained directly with six to eight data points.

Derivation of the Michaelis–Menten Equation

The generalized statement of the mechanism of enzyme action is

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad \textbf{(4.10)}$$

If we assume that the rate of formation of the ES complex is balanced by its rate of breakdown (the steady-state assumption), then we can write

$$v_{formation} = k_1[S][E]$$
$$v_{breakdown} = k_2[ES] + k_3[ES] = [ES](k_2 + k_3)$$

If we set the rate of formation equal to the rate of breakdown, then

$$k_1[S][E] = [ES](k_2 + k_3)$$

After dividing both sides of the equation by k_1, we have

$$[S][E] = [ES]\left[\frac{k_2 + k_3}{k_1}\right] \qquad \textbf{(4.14)}$$

If we now define the ratio of the rate constants $(k_2 + k_3)/k_1$ as K_m, the Michaelis constant, and substitute it into Eq. 4.14, then

$$[S][E] = [ES]K_m \qquad \textbf{(4.15)}$$

Since [E] is equal to the free enzyme, we must express its concentration in terms of the total enzyme added to the system minus any enzyme in the [ES] complex; that is,

$$[E] = ([E_t] - [ES])$$

Upon substitution of the equivalent expression for [E] into Eq. 4.15 we have

$$[S]([E_t] - [ES]) = [ES]K_m$$

Dividing through by [S] yields

$$[E_t] - [ES] = \frac{[ES]K_m}{[S]}$$

and dividing through by [ES] yields

$$\frac{[E_t]}{[ES]} - 1 = \frac{K_m}{[S]} \quad \text{or} \quad \frac{[E_t]}{[ES]} = \frac{K_m}{[S]} + 1 = \frac{K_m + [S]}{[S]} \qquad \textbf{(4.16)}$$

We now need to obtain an alternative expression for $[E_t]/[ES]$, since [ES] cannot be measured easily, if at all. When the enzyme is saturated with substrate all the enzyme will be in the ES complex, and none will be free, $[E_t] = [ES]$, and the velocity observed will be the maximum possible; therefore, $V_{max} = k_3$. When $[E_t]$ is not equal to [ES], $v = k_3[ES]$. From these two expressions we can obtain the ratio of $[E_t]/[ES]$; that is,

$$\frac{[E_t]}{[ES]} = \frac{V_{max}/k_3}{v/k_3} = \frac{V_{max}}{v} \qquad \textbf{(4.17)}$$

Substituting this value of $[E_t]/[ES]$ into Eq. 4.16 yields a form of the Michaelis–Menten equation:

$$\frac{V_{max}}{v} = \frac{K_m + [S]}{[S]}$$

or

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

An Enzyme Catalyzes Both Forward and Reverse Directions of a Reversible Reaction

As indicated previously, enzymes do not alter the equilibrium constant of a reaction; consequently, in the reaction

$$S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P$$

the direction of flow of material, either in the forward or the reverse direction, will depend on the concentration of S relative to P and the equilibrium constant of the reaction. Since enzymes catalyze the forward and reverse reactions, a problem may arise if product has an affinity for the enzyme that is similar to that of substrate. In this case the product can easily rebind to the active site of the enzyme and will compete with the substrate for that site. In such cases the product inhibits the reaction as concentration of product increases. The Lineweaver–Burk plot will not be linear in those cases where the enzyme is susceptible to **product inhibition.** If the subsequent enzyme in a metabolic pathway removes the product rapidly, then product inhibition should not occur.

Product inhibition in a metabolic pathway provides a limited means of controlling or modulating flux of substrates through the pathway. As the end product of a pathway increases, each intermediate will also increase by mass action. If one or more enzymes are particularly sensitive to product inhibition, output of end product of the pathway will be suppressed. Reversibility of a pathway or a particular enzyme-catalyzed reaction is dependent on the rate of product removal. If the end product is quickly removed, then the pathway may be physiologically unidirectional.

Multisubstrate Reactions Follow Either a Ping–Pong or Sequential Mechanism

Most enzymes utilize more than one substrate, or act upon one substrate plus a coenzyme and generate one or more products. In any case, a K_m must be determined for each substrate and coenzyme involved in the reaction when establishing an enzyme assay.

Mechanistically, enzyme reactions are divided into two major categories, ping–pong or sequential. There are many variations on these major mechanisms. The **ping–pong mechanism** can be represented as follows:

$$E + A \longrightarrow EA \longrightarrow \overset{\overset{P_1}{\uparrow}}{E'} \longrightarrow \overset{\overset{B}{\downarrow}}{E'B} \longrightarrow P_2 + E$$

where substrate A reacts with E to produce product P_1, which is released before the second substrate B binds to the modified enzyme E . Substrate B is then converted to product P_2 and the enzyme is regenerated. A good example of this mechanism is the transaminase-catalyzed reaction (see p. 448) in which the α-amino group of amino $acid_1$ is transferred to the enzyme and the newly formed α-keto $acid_1$ is released, as the first product, followed by the binding of the acceptor α-keto $acid_2$ and release of amino $acid_2$. This reaction is outlined in Figure 4.17.

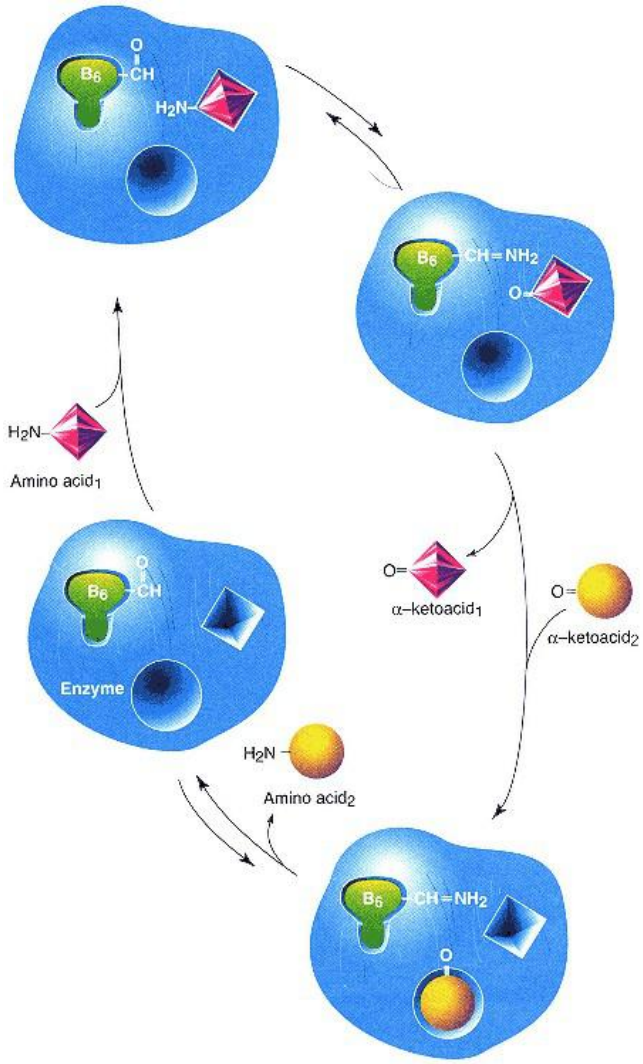


Figure 4.17
Schematic representation of the trans-aminase reaction mechanism: an example of a ping-pong mechanism.
Enzyme-bound pyridoxal phosphate (vitamin B_6 coenzyme) accepts the -amino group from the first amino acid (AA_1), which is then released from the enzyme as an -keto acid. The acceptor -keto acid (AA_2) is then bound to the enzyme, and the bound amino group is transferred to it, forming a new amino acid, which is then released from the enzyme. The terms "oxy" and "keto" are used interchangeably.

CLINICAL CORRELATION 4.3

Mutation of a Coenzyme-Binding Site Results in Clinical Disease

Cystathioninuria is a genetic disease in which γ-cystathionase is either deficient or inactive. Cystathionase catalyzes the reaction:

Deficiency of the enzyme leads to accumulation of cystathionine in the plasma. Since cystathionase is a pyridoxal phosphate-dependent enzyme, vitamin B_6 was administered to patients whose fibroblasts contained material that cross-reacted with antibody against cystathionase. Many responded to B_6 therapy with a fall in plasma levels of cystathionine. These patients produce the apoenzyme that reacted with the antibody. In one patient the enzyme activity was undetectable in fibroblast homogenates but increased to 31% of normal with the addition of 1 mM of pyridoxal phosphate to the assay mixture. It is thought that the K_m for pyridoxal phosphate binding to the enzyme was increased because of a mutation in the binding site. Activity is partially restored by increasing the concentration of coenzyme. Apparently these patients require a higher steady-state concentration of coenzyme to maintain γ-cystathionase activity.

Pascal, T. A., Gaull, G. E., Beratis, N. G., Gillam, B. M., Tallan, H. H., and Hirschhorn, K. Vitamin B_6-responsive and unresponsive cystathionuria: two variant molecular forms. *Science* 190:1209, 1975.

In the **sequential mechanism,** if two substrates A and B can bind in any order, it is a random mechanism; if binding of A is required before B can be bound, then it is an ordered mechanism. In either case the reaction is bimolecular; that is, both A and B must be bound before reaction occurs. Examples of these mechanisms are found among the dehydrogenases in which the second substrate is a coenzyme (NAD^+, FAD, etc.; see p. 143). Release of products may or may not be ordered in either mechanism.

4.4— Coenzymes: Structure and Function

Coenzymes are small organic molecules, often derivatives of vitamins, that function with the enzyme in the catalytic process. Often the coenzyme has an affinity for the enzyme that is similar to that of the substrate; consequently, the coenzyme can be considered to be a second substrate. In some cases, the coenzyme is covalently bound to the apoenzyme and functions at or near the active site in catalysis. In other enzymes the role of the coenzyme falls between these two extremes.

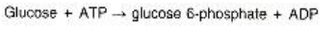
Several coenzymes are derived from the B vitamins. Vitamin B_6, pyridoxine, requires little modification to form the active coenzyme, pyridoxal phosphate (see p. 1121). Clinical Correlation 4.3 points out the importance of the coenzyme-binding site and how alterations in this site cause metabolic dysfunction.

In contrast to vitamin B_6, niacin requires major alteration in mammalian cells to form a coenzyme, as outlined in Section 12.9.

The structures and functions of the coenzymes of only two B vitamins, niacin and riboflavin, and of ATP are discussed in this chapter. The structures and functions of coenzyme A (CoA) (see p. 514), thiamine (see p. 1119), biotin, and vitamin B_{12} are included in those chapters dealing with enzymes dependent on the given coenzyme for activity.

Adenosine Triphosphate May Be a Second Substrate or a Modulator of Activity

Adenosine triphosphate (ATP) often functions as a second substrate but can also serve as a cofactor in modulation of the activity of specific enzymes. This compound is central in biochemistry (Figure 4.18) and it is synthesized *de novo* in all mammalian cells. The nitrogenous heterocyclic ring is adenine. The combination of the base, adenine, plus ribose is known as adenosine; hence ATP is adenosine that has at the 5 -hydroxyl a triphosphate. The biochemically functional end is the reactive triphosphate. The terminal phosphate–oxygen bond has a high free energy of hydrolysis, which means that the phosphate can be transferred from ATP to other acceptor groups. For example, as a cosubstrate ATP is utilized by the kinases for the transfer of the terminal phosphate to various acceptors. A typical example is the reaction catalyzed by **glucokinase:**



ADP is adenosine diphosphate.

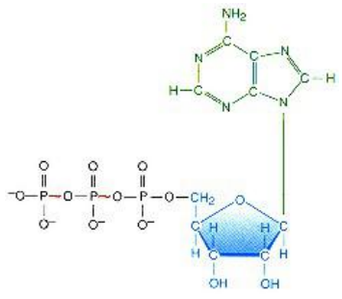


Figure 4.18
Adenosine triphosphate (ATP).

ATP also serves as a modulator of the activity of some enzymes. These enzymes have binding sites for ATP, occupancy of which changes the affinity or reactivity of the enzyme toward its substrates. In these cases, ATP acts as an **allosteric effector** (see p. 151).

NAD and NADP are Coenzyme Forms of Niacin

Niacin is pyridine-3-carboxylic acid. It is converted to two coenzymes involved in oxidoreductase reactions. They are **NAD (nicotinamide adenine dinucleo-**

tide) and **NADP (nicotinamide adenine dinucleotide phosphate).** The abbreviations NAD and NADP are convenient to use when referring to the coenzymes regardless of their state of oxidation or reduction. NAD^+ and $NADP^+$ represent the oxidized forms, and NADH and NADPH represent the reduced forms. Some dehydrogenases are specific for NADP and others for NAD; some function with either coenzyme. This arrangement allows for specificity and control over dehydrogenases that reside in the same subcellular compartment.

NAD^+ consists of adenosine and *N*-ribosyl-nicotinamide linked by a pyrophosphate linkage between the 5 -OH groups of the two ribosyl moieties (Figure 4.19). NADP differs structurally from NAD in having an additional phosphate esterified to the 2 -OH group of the adenosine moiety. Both coenzymes function as intermediates in transfer of two electrons between an electron donor and an acceptor. The donor and acceptor need not be involved in the same metabolic pathway. Thus the reduced form of these nucleotides acts as a common "pool" of electrons that arise from many oxidative reactions and can be used for various reductive reactions.

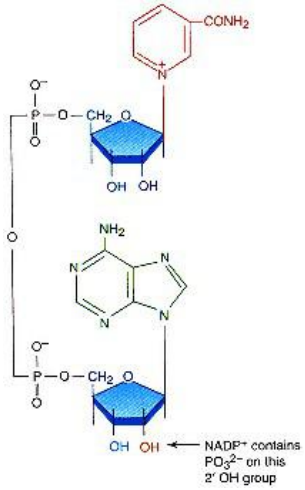


Figure 4.19
Nicotinamide adenine dinucleotide (NAD^+).

The adenine, ribose, and pyrophosphate components of NAD are involved in binding of NAD to the enzyme. Enzymes requiring NADP do not have a conserved aspartate residue present in the NAD-binding site. If the aspartate were present, a charge–charge interaction between the negative charged aspartate and the 2 -phosphate of NADP would prevent binding. Since there is no negatively charged phosphate on the 2 -OH in NAD, there is discrimination between NAD and NADP binding. The nicotinamide reversibly accepts and donates two electrons at a time. It is the active center of the coenzyme. In oxidation of deuterated ethanol by alcohol dehydrogenase, NAD^+ accepts two electrons and the deuterium from the ethanol. The other hydrogen is released as a H^+ (Figure 4.20).

The binding of NAD^+ to the enzyme surface confers a chemically recognizable "top side" and "bottom side" to the planar nicotinamide. The former is known as the **A** face and the latter as the **B** face. In the case of alcohol dehydrogenase, the proton or deuterium ion that serves as a tracer is added to the **A** face. Other dehydrogenases utilize the **B** face. This particular effect demonstrates how enzymes can induce **stereospecificity** in chemical reactions by virtue of the asymmetric binding of coenzymes and substrates.

FMN and FAD Are Coenzyme Forms of Riboflavin

The two coenzyme forms of riboflavin are **FMN (flavin mononucleotide)** and **FAD (flavin adenine dinucleotide)**. The vitamin riboflavin consists of the heterocyclic ring, isoalloxazine (flavin) connected through N-10 to the alcohol ribitol (Figure 4.21). FMN has a phosphate esterified to the 5 -OH group of ribitol. FAD is structurally analogous to NAD in having adenosine linked by a pyrophosphate linkage to a heterocyclic ring, in this case riboflavin (Figure 4.22). Both FAD and FMN function in oxidoreduction reactions by accepting and donating $2e^-$ in the isoalloxazine ring. A typical example of FAD participation in an enzyme reaction is the oxidation of succinate to fumarate by succinate

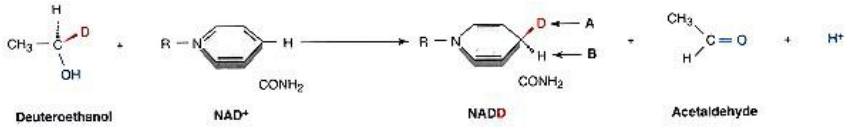


Figure 4.20
Stereo specific transfer of deuterium from deuterated ethanol to NAD^+.

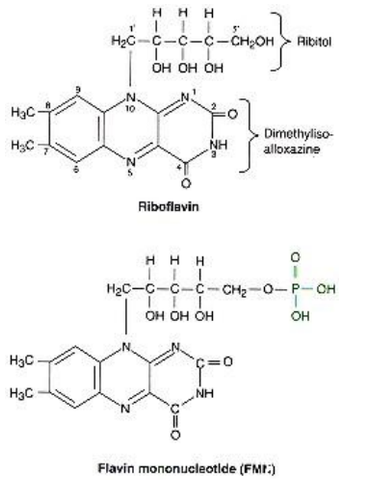


Figure 4.21
Riboflavin and flavin mononucleotide.

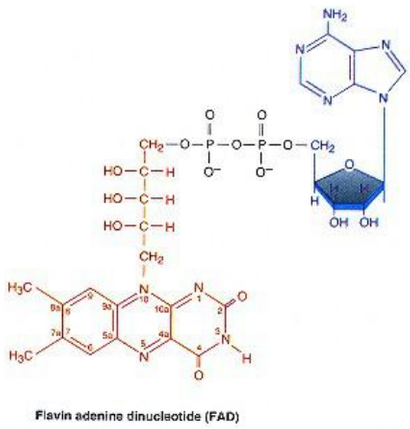


Figure 4.22
Flavin adenine dinucleotide (FAD).

dehydrogenase (see p. 236) (Figure 4.23). In some cases, these coenzymes are $1e^-$ acceptors, which lead to flavin semiquinone formation (a free radical).

Flavin coenzymes tend to be bound much tighter to their apoenzymes than the niacin coenzymes and often function as prosthetic groups rather than as cofactors.

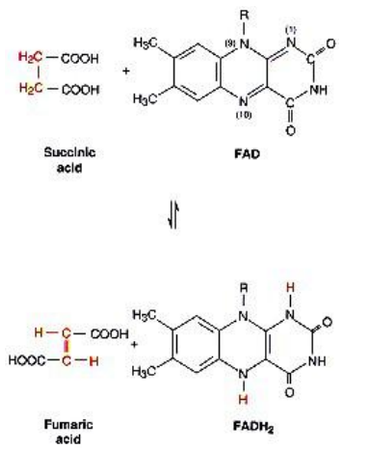


Figure 4.23
FAD as a coenzyme in the succinic dehydrogenase reaction.

Metal Cofactors Have Various Functions

Metals are not coenzymes in the same sense as FAD, FMN, NAD^+, and $NADP^+$ but are required as cofactors in approximately two-thirds of all enzymes. Metals participate in enzyme reactions by acting as Lewis acids and by various modes of chelate formation. **Chelates** are organometallic coordination complexes. A good example of a chelate is the complex between iron and protoporphyrin IX to form a heme (see p. 115). Metals that act as Lewis acid catalysts are found among the transition metals like Zn, Fe, Mn, and Cu, which have empty *d* electron orbitals that act as electron sinks. The alkaline earth metals such as K and Na do not possess this ability. A good example of a metal functioning as a Lewis acid is found in **carbonic anhydrase**, a zinc enzyme that catalyzes the reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

The first step (Figure 4.24) can be visualized as the *in situ* generation of a proton and a hydroxyl group from water binding to the zinc (Lewis acid function of zinc). The proton and hydroxyl group are then added to the carbon dioxide and carbonic acid is released. Actually, the reactions presented in sequence may occur in a concerted fashion, that is, all at one time.

Metals can also promote catalysis either by binding substrate and promoting electrophilic catalysis at the site of bond cleavage or by stabilizing intermediates in the reaction pathway. In the case of **carboxypeptidase** and **thermolysin, zinc proteases** with identical active sites, the zinc functions to generate a hydroxyl group from water, and then to stabilize the transition state resulting from attack of the hydroxyl on the peptide bond. Figure 4.25 depicts the generation of the active-site hydroxyl by zinc. As shown, Glu 270 functions as

a base in plucking the proton from water. Stabilization of the tetrahedral transition state by zinc is shown in Figure 4.26. The positive zinc provides a counterion to stabilize the negative oxygens on the tetrahedral carbon.

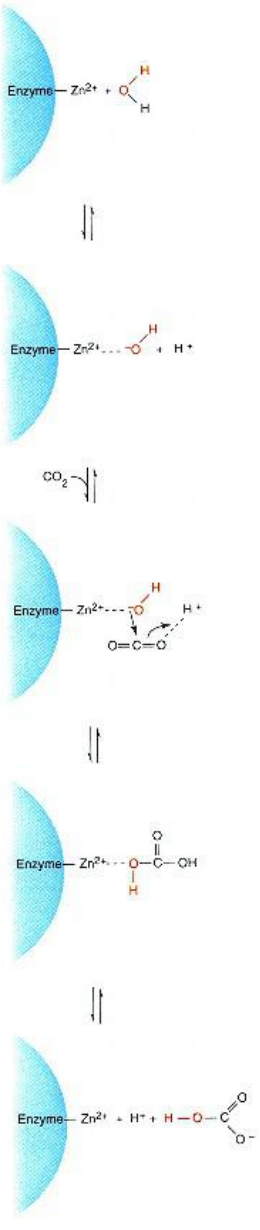


Figure 4.24
Zinc functions as a
Lewis acid in carbonic anhydrase.

Role of the Metal As a Structural Element

The function of a metal as a Lewis acid in carbonic anhydrase and carboxypeptidase requires chelate formation. Various modes of chelation occur between metal, enzyme, and substrate that are structural in nature, but in which no acid catalysis occurs.

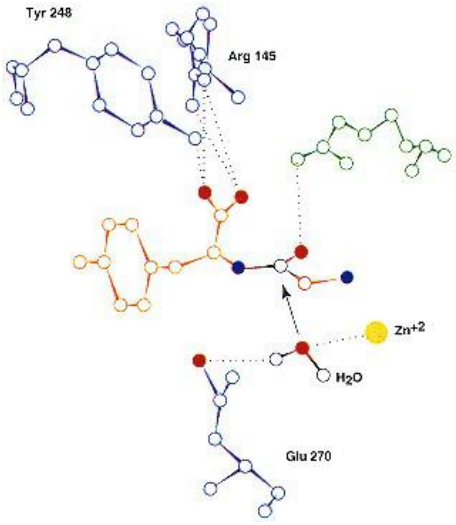


Figure 4.25
Zinc in the mechanism of reaction of carboxypeptidase A.
Enzyme -bound zinc generates a hydroxyl nucleophile from bound water, which attacks the carbonyl of the peptide bond as indicated by the arrows. Glu 270 assists by pulling the proton from the zinc-bound water.
Redrawn from Lipscomb, W. N. *Robert A. Welch Found. Conf. Chem. Res.* 15:140,1971.

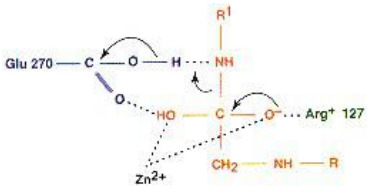


Figure 4.26
Stabilization of the transition state of the tetrahedral intermediate by zinc.
Positive charge on the zinc stabilizes the negative charge that develops on the oxygens of the tetrahedral carbon in the transition state. The tetrahedral intermediate then collapses as indicated by the arrows, resulting in breakage of the peptide bond.

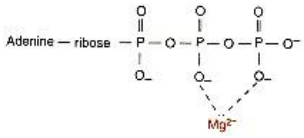


Figure 4.27
Mg^{2+}–ATP.

In several kinases, creatine kinase being the best example, the true substrate is not ATP but Mg^{2+}–ATP (Figure 4.27). In this case, Mg^{2+} does not interact directly with the enzyme. It may serve to neutralize the negative charge density on ATP and facilitate binding to the enzyme. Ternary complexes of this conformation are known as "substrate-bridged" complexes and can be schematically represented as Enz–S–M. A hypothetical scheme for the binding of Mg^{2+}–ATP and glucose in the active site of hexokinase is presented in Figure 4.28. All kinases except muscle pyruvate kinase and phosphoenolpyruvate carboxykinase are substrate-bridged complexes. In pyruvate kinase, Mg^{2+} chelates ATP to the enzyme as shown in Figure 4.29. Absence of the metal cofactor results in failure of ATP to bind to the enzyme. Enzymes of this class are "metal-bridged" ternary complexes, Enz–S–M. All **metalloenzymes** are of this type and contain a tightly bound transition metal such as Zn^{2+} or Fe^{2+}. Several enzymes that catalyze enolization and elimination reactions are metal-bridged complexes.

In addition to the role of binding enzyme and substrate, metals may also bind directly to the enzyme to stabilize it in the active conformation or perhaps to induce the formation of a binding site or active site. Not only do the strongly chelated metals like Mn^{2+} play a role in this regard, but the weakly bound alkali metals (Na^+ or K^+) are also important. In **pyruvate kinase**, K^+ induces an initial conformation change, which is necessary, but not sufficient, for ternary complex formation. Upon substrate binding, K^+ induces a second conformational change to the catalytically active ternary complex as indicated in Figure 4.29. Thus Na^+ and K^+ stabilize the active conformation of the enzyme but are passive in catalysis.

Role of Metals in Oxidation and Reduction

Iron–sulfur proteins, often referred to as nonheme iron proteins, are a unique class of metalloenzymes in which the active center consists of one or more clusters of sulfur-bridged iron chelates. The structures are presented on page 252. In some cases the sulfur comes only from cysteine and in others from both cysteine and free ionic sulfur. The free sulfur is released as hydrogen

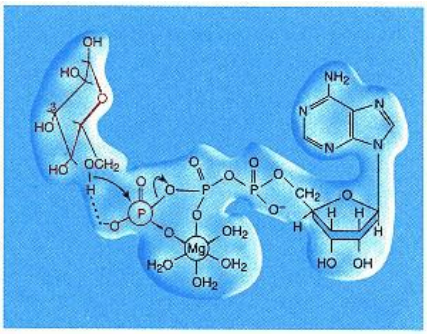


Figure 4.28
Role of Mg^{2+} as a substrate-bridged complex in the active site of the kinases.
In hexokinase the terminal phosphate of ATP is transferred to glucose, yielding glucose 6-phosphate. Mg^{2+} coordinates with the ATP to form the true substrate and in addition may labilize the terminal P–O bond of ATP to facilitate transfer of the phosphate to glucose. There are specific binding sites (light blue) on the enzyme (darker blue) for glucose (upper left) in red as well as the adenine and ribose moieties of ATP (black).

sulfide upon acidification. These nonheme iron enzymes have reasonably low reducing potentials (E_0') and function in electron-transfer reactions (see p. 251).

Cytochromes are heme iron proteins that function as cosubstrates for their respective reductases (see p. 252). Iron in hemes of cytochromes undergoes reversible le^- transfers. Heme is bound to the apoprotein by coordination of an amino acid side chain to iron of heme. Thus the metal serves not only a structural role but also participates in the chemical event.

Metals, specifically copper and iron, also have a role in activation of molecular oxygen. Copper is an active participant in several oxidases and hydroxylases. For example, **dopamine β-hydroxylase** catalyzes the introduction of one oxygen atom from O_2 into dopamine to form norepinephrine (Figure 4.30). The active enzyme contains one atom of cuprous ion that reacts with oxygen to form an activated oxygen–copper complex. The copper–hydroperoxide complex shown in Figure 4.30 is thought to be converted to a copper(II)–O^- species that serves as the "active oxygen" in the hydroxylation of DOPA. In other metalloenzymes other species of "active oxygen" are generated and used for hydroxylation.

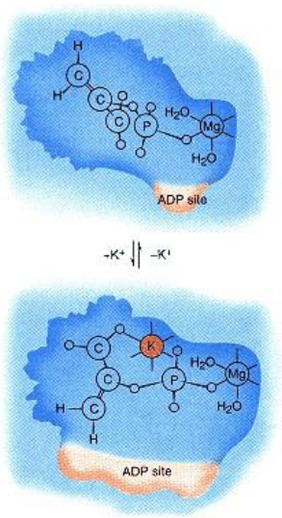


Figure 4.29
Model of the role of K^+ in the active site of pyruvate kinase.
Pyruvate kinase catalyzes the reaction: phosphoenolpyruvate + ADP ATP + pyruvate. Initial binding of K^+ induces conformational changes in the kinase, which result in increased affinity for phosphoenolpyruvate. In addition, K^+ orients the phosphoenolpyruvate in the correct position for transfer of its phosphate to ADP, the second substrate. Mg^{2+} coordinates the substrate to the enzyme active site.
Modified with permission from Mildvan, A. S. *Annu. Rev. Biochem.* 43:365, 1974.

4.5— Inhibition of Enzymes

Mention was made of product inhibition of enzyme activity and how an entire pathway can be controlled or modulated by this mechanism (see p. 140). In addition to inhibition by the immediate product, products of other enzymes can also inhibit or activate a particular enzyme. Much of current drug therapy is based on inhibition of specific enzymes by a substrate analog.

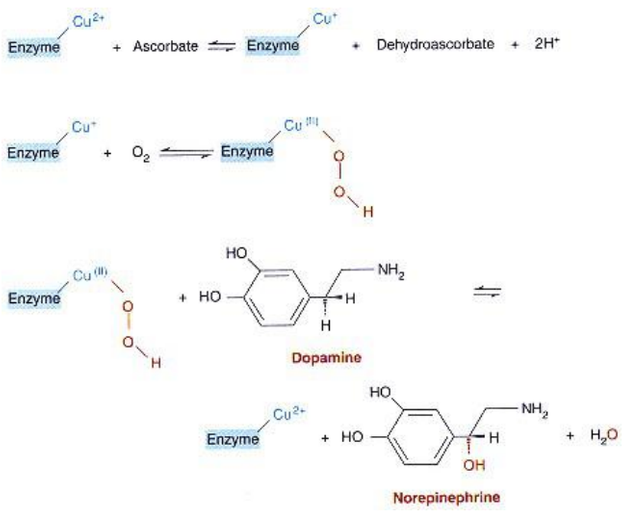


Figure 4.30
Role of copper in activation of molecular oxygen by dopamine hydroxylase.
The normal cupric form of the enzyme is not reactive with oxygen but on reduction by the cosubstrate, ascorbate, generates a reactive enzyme–copper bound oxygen radical that then reacts with dopamine to form norepinephrine and an inactive cupric enzyme.

There are three major classes of inhibitors: competitive, noncompetitive, and uncompetitive.

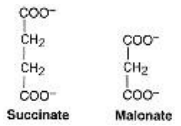


Figure 4.31
Substrate and inhibitor of succinate dehydrogenase.

Competitive Inhibition May Be Reversed by Increased Substrate

Competitive inhibitors are inhibitors whose action can be reversed by increasing amounts of substrate. Competitive inhibitors are structurally similar to the substrate and bind at the substrate-binding site, thus competing with the substrate for the enzyme. Once bound, the enzyme cannot convert the inhibitor to products. Increasing substrate concentrations will displace the reversibly bound inhibitor by the law of mass action. For example, in the **succinate dehydrogenase** reaction, malonate is structurally similar to succinate and is a competitive inhibitor (Figure 4.31).

Since substrate and inhibitor compete for the same binding site, the K_m for the substrate shows an apparent increase in the presence of inhibitor. This can be seen in a double-reciprocal plot as a shift in the x-intercept ($-1/K_m$) and in the slope of the line (K_m/V_{max}). If we first establish the velocity at several levels of substrate and then repeat the experiment with a given but constant amount of inhibitor at various substrate levels, two different straight lines will be obtained (Figure 4.32). V_{max} does not change; hence the intercept on the y-axis remains the same. In the presence of inhibitor, the x-intercept is no longer the negative reciprocal of the true K_m, but of an apparent value, $K_{m,app}$ where

$$K_{m,app} = K_m \cdot \left(1 + \frac{[I]}{K_I}\right)$$

Thus the inhibitor constant, K_I, can be determined from the concentration of inhibitor used and the K_m, which was obtained from the x-intercept of the line obtained in the absence of inhibitor.

Noncompetitive Inhibitors Do Not Prevent Substrate from Binding

A **noncompetitive inhibitor** binds at a site other than the substrate-binding site. Inhibition is not reversed by increasing concentration of substrate. Both binary (EI) and ternary (EIS) complexes form, which are catalytically inactive

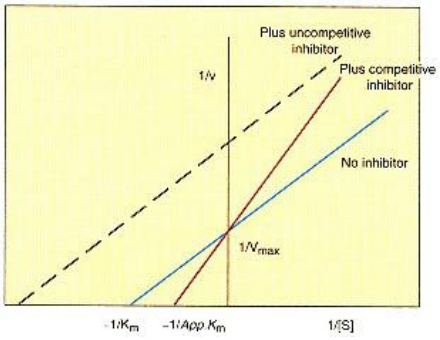


Figure 4.32
Double-reciprocal plots for competitive and uncompetitive inhibition.
A competitive inhibitor binds at the substrate-binding site and effectively increases the K_m for the substrate. An uncompetitive inhibitor causes an equivalent shift in both V_{max} and K_m, resulting in a line parallel to that given by the uninhibited enzyme.

and are therefore dead-end complexes. A noncompetitive inhibitor behaves as though it were removing active enzyme from the solution, resulting in a decrease in V_{max}. This is seen graphically in the double-reciprocal plot (Figure 4.33), where K_m does not change but V_{max} does change. Inhibition can often be reversed by exhaustive dialysis of the inhibited enzyme provided that the inhibitor has not reacted covalently with the enzyme as discussed under irreversible inhibitors.

An **uncompetitive inhibitor** binds only with the ES form of the enzyme in the case of a one-substrate enzyme. The result is an apparent equivalent change in K_m and V_{max}, which is reflected in the double-reciprocal plot as a line parallel to that of the uninhibited enzyme (Figure 4.32). In the case of multisubstrate enzymes the interpretation is complex and will not be considered further.

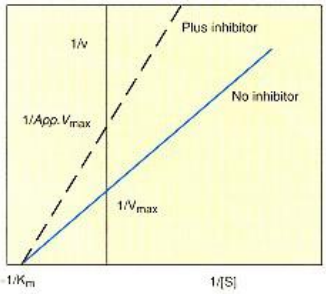


Figure 4.33
Double-reciprocal plot for an enzyme subject to reversible noncompetitive inhibition.
A noncompetitive inhibitor binds at a site other than the substrate-binding site; therefore the effective K_m does not change, but the apparent V_{max} decreases.

Irreversible Inhibition Involves Covalent Modification of an Enzyme Site

When covalent modification occurs at the binding site or the active site, inhibition will not be reversed by dialysis unless the linkage is chemically labile like that of an ester or thioester. The active-site thiol group in glyceraldehyde-3-phosphate dehydrogenase reacts with ***p*-chloromercuribenzoate** to form a mercuribenzoate adduct of the enzyme (Figure 4.34). Such adducts are not reversed by dialysis or by addition of substrate. Double-reciprocal plots show the characteristic pattern for noncompetitive inhibition (Figure 4.33).

Many Drugs Are Enzyme Inhibitors

Most modern drug therapy is based on the concepts of enzyme inhibition that were described in the previous section. Drugs are designed to inhibit a specific enzyme in a metabolic pathway. This application is most easily appreciated with antiviral, antibacterial, and antitumor drugs that are administered to the patient under conditions of limited toxicity. Such toxicity is often unavoidable because, with the exception of cell wall biosynthesis in bacteria, there are few critical metabolic pathways that are unique to tumors, viruses, or bacteria. Hence drugs that kill these organisms will often kill host cells. The one characteristic that can be taken advantage of is the comparatively short generation time of the undesirable organisms. They are much more sensitive to antimetabolites and in particular those that inhibit enzymes involved in replication. Antimetabolites are compounds with some structural difference from the natural substrate. In subsequent chapters, numerous examples of **antimetabolites** will be described. Here we will present a few examples that illustrate the concept.

Sulfa Drugs

Modern chemotherapy had its beginning in compounds of the general formula $R–SO_2–NHR$. Sulfanilamide, the simplest member of the class, is an antibacterial agent because of its competition with ***p*-aminobenzoic acid** (PABA), which

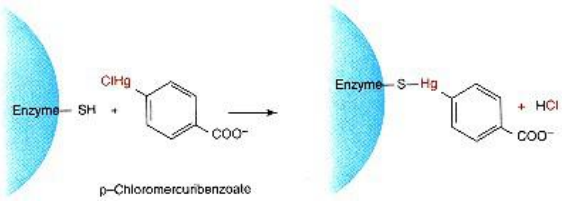


Figure 4.34
Enzyme inhibition by a covalent modification of an active center cysteine.

is required for bacterial growth. Structures of these compounds are shown in Figure 4.35.

Bacteria cannot absorb folic acid, a required vitamin for the host, but must synthesize it. Since sulfanilamide is a structural analog of *p*-aminobenzoate, the bacterial dihydropteroate synthetase is tricked into making an intermediate, containing sulfanilamide, that cannot be converted to folate. Figure 4.36*b* shows the fully reduced or coenzyme form of folate. Thus the bacterium is starved of the required folate and cannot grow or divide. Since humans require folate from dietary sources, the sulfanilamide is not harmful at the doses that kill bacteria.

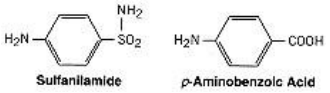


Figure 4.35
Structure of *p*-aminobenzoic acid and sulfanilamide, a competitive inhibitor.

Methotrexate

Biosynthesis of purines and pyrimidines, heterocyclic bases required for synthesis of RNA and DNA, requires folic acid, which serves as a coenzyme in transfer of one-carbon units from various amino acid donors (see p. 460). Methotrexate (Figure 4.36*a*), a structural analog of folate, has been used with great success in childhood leukemia. Its mechanism of action is based on competition with dihydrofolate for dihydrofolate reductase. It binds 1000-fold more strongly than the natural substrate and is a powerful competitive inhibitor of the enzyme. The synthesis of thymidine monophosphate stops in the presence of methotrexate because of failure of the one-carbon transfer reaction. Since cell division depends on thymidine monophosphate as well as the other nucleotides, the leukemia cell cannot multiply. One problem is that rapidly dividing human cells such as those in bone marrow and intestinal mucosa are sensitive to the drug for the same reasons. Also, prolonged usage leads to amplification of the gene for dihydrofolate reductase, with increased levels of the enzyme and preferential growth of methotrexate-resistant cells.

Neoclassical Antimetabolites

A nonclassical antimetabolite is a substrate for an enzyme that upon action of the enzyme generates a highly reactive species. This species forms a covalent adduct with an amino acid at the active site, leading to irreversible inactivation of the enzyme. These inhibitors are referred to as suicide substrates and are very specific. Another group of inhibitors contains a reactive functional group. For example, the compound shown in Figure 4.37 is an irreversible inhibitor of **dihydrofolate reductase** because it is specifically bound at the active site and the reactive benzylsulfonyl fluoride is positioned to react with a serine hydroxyl group in the substrate-binding site. Covalent binding of this substrate analog to the enzyme prevents binding of the normal substrate and inhibits the enzyme.

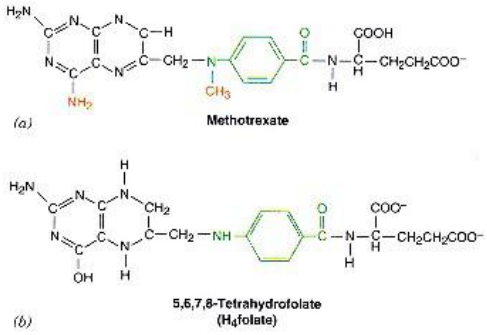


Figure 4.36
Methotrexate
(4-amino-N^{10}-methyl folic acid) and tetrahydrofolic acid.
Contribution from *p*-aminobenzoate is shown in green.

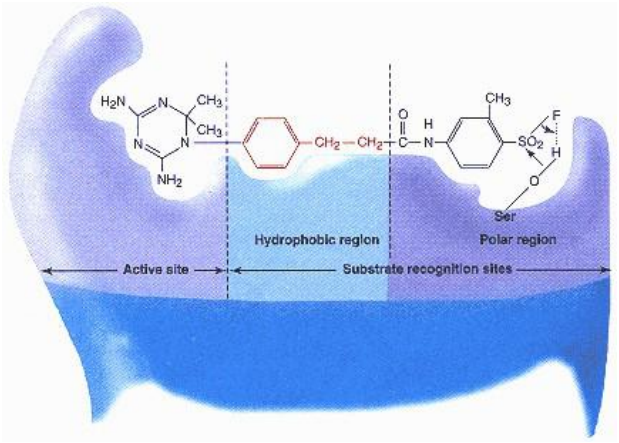


Figure 4.37
Site-directed inactivation of tetrahydrofolate reductase.
The irreversible inhibitor, a substituted dihydrotriazine, structurally resembles dihydrofolate and binds specifically to the dihydrofolate site on dihydrofolate reductase. The triazine portion of the inhibitor resembles the pterin moiety and therefore binds to the active site. The ethylbenzene group (in red) binds to the hydrophobic site normally occupied by the p-aminobenzoyl group. The reactive end of the inhibitor contains a reactive sulfonyl fluoride that forms a covalent linkage with a serine hydroxyl on the enzyme surface. Thus this inhibitor irreversibly inhibits the enzyme by blocking access of dihydrofolate to the active site.

Other Antimetabollites

Two other analogs of the purines and pyrimidines will be mentioned to emphasize the structural similarity of chemotherapeutic agents to normal substrates. **Fluorouracil** (Figure 4.38) is a thymine analog in which the ring-bound methyl is substituted by fluorine. The deoxynucleotide of this compound is an irreversible inhibitor of thymidylate synthetase. **6-Mercaptopurine** (Figure 4.38) is an analog of hypoxanthine, adenine, and guanine, which is converted to the 6-mercaptopurine nucleotide in cells. This nucleotide is a broad spectrum antimetabolite because of its competition in reactions involving adenine and guanine nucleotides. The antimetabolites discussed relate to purine and pyrimidine metabolism but the general concepts can be applied to any enzyme or metabolic pathway.

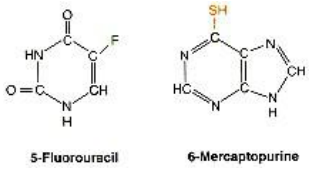


Figure 4.38
Structures of two antimetabolites.

4.6—
Allosteric Control of Enzyme Activity

Allosteric Effectors Bind at Sites Different from Substrate-Binding Sites

Although the substrate-binding and active site of an enzyme are well-defined structures, the activity of many enzymes can be modulated by ligands acting in ways other than as competitive or noncompetitive inhibitors. A **ligand** is any molecule that is bound to a macromolecule; the term is not limited to small organic molecules, such as ATP, but includes low molecular weight proteins. Ligands can be activators, inhibitors, or even the substrates of enzymes. Those ligands that change enzymatic activity, but are unchanged as a result of enzyme action, are referred to as effectors, modifiers, or modulators. Most of the enzymes subject to modulation by ligands are rate-determining enzymes in metabolic pathways. To appreciate the mechanisms by which metabolic pathways are controlled, the principles governing the allosteric and cooperative behavior of individual enzymes must be understood.

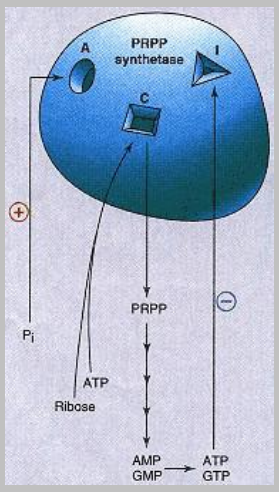
Enzymes that respond to modulators have additional site(s) known as **allosteric site(s).** Allosteric is derived from the Greek root *allo*, meaning "the other." An allosteric site is a unique region of the enzyme quite different from the substrate-binding site. The existence of allosteric sites is illustrated in Clin. Corr. 4.4. The ligands that bind at the allosteric site are called allosteric effectors

or modulators. Binding of an allosteric effector causes a conformational change of the enzyme so that the affinity for the substrate or other ligands also changes. Positive (+) allosteric effectors increase the enzyme affinity for substrate or other ligand. The reverse is true for negative (—) allosteric effectors. The allosteric site at which the positive effector binds is referred to as an activator site; the negative effector binds at an inhibitory site.

CLINICAL CORRELATION 4.4

A Case of Gout Demonstrates the Difference Between an Allosteric and Substrate-Binding Site

The realization that allosteric inhibitory sites are separate from allosteric activator sites as well as from the substrate-binding and the catalytic sites is illustrated by a study of a gouty patient whose red blood cell PRPP level was increased (see Clin. Corr. 4.1). It was found that the patient's PRPP synthetase had normal K_m and V_{max} values, and sensitivity to activation by phosphate. The increased PRPP levels and hyperuricemia arose because the end products of the pathway (ATP, GTP) were not able to inhibit the synthetase through the allosteric inhibitory site (I). It was suggested that a mutation in the inhibitory site or in the coupling mechanism between the inhibitory and catalytic site led to failure of the feedback control mechanism.



Sperling, O., Perksy-Brosh, S., Boen, P., and DeVries, A. Human erythrocyte phosphoribosyl-pyrophosphate synthetase mutationally altered in regulatory properties. *Biochem. Med.* 7: 389, 1973.

Allosteric enzymes are divided into two classes based on the effect of the allosteric effector on the K_m and V_{max}. In the **K class** the effector alters the K_m but not V_{max}, whereas in the **V class** the effector alters V_{max} but not K_m. K class enzymes give double-reciprocal plots like those of competitive inhibitors (Figure 4.32) and V class enzymes give double-reciprocal plots like those of noncompetitive inhibitors (Figure 4.33). The terms competitive and noncompetitive are inappropriate for allosteric enzyme systems because the mechanism of the effect of an allosteric inhibitor on a V or K enzyme is different from that of a simple competitive or noncompetitive inhibitor. For example, in the K class, the negative effector binding at an allosteric site affects the affinity of the substrate-binding site for the substrate, whereas in simple competitive inhibition the inhibitor competes directly with substrate for the site. In V class enzymes, positive and negative allosteric modifiers increase or decrease the rate of breakdown of the ES complex to products. The catalytic rate constant, k_3, is affected and not the substrate-binding constant. There are a few enzymes in which both K_m and V_{max} are affected.

In theory, a monomeric enzyme can undergo an allosteric transition in response to a modulating ligand. In practice, only two monomeric allosteric enzymes are known, ribonucleoside diphosphate reductase and pyruvate-UDP-*N*-acetylglucosamine transferase. Most allosteric enzymes are **oligomeric;** that is, they consist of several subunits. Identical subunits are designated as **protomers,** and each protomer may consist of one or more polypeptide chains. As a consequence of the oligomeric nature of allosteric enzymes, binding of ligand to one protomer can affect the binding of ligands on the other protomers in the oligomer. Such ligand effects are referred to as **homotropic** interactions. Transmission of the homotropic effects between protomers is one aspect of cooperativity, considered later. Substrate influencing substrate, activator influencing activator, or inhibitor influencing inhibitor binding are homotropic interactions. Homotropic interactions are almost always positive.

A **heterotropic** interaction is the effect of one ligand on the binding of a different ligand. For example, the effect of a negative effector on the binding of substrate or on binding of an allosteric activator are heterotropic interactions. Heterotropic interactions can be positive or negative and can occur in monomeric allosteric enzymes. Heterotropic and homotropic effects in oligomeric enzymes are mediated by cooperativity between subunits.

Based on the foregoing descriptions of allosteric enzymes, two models are pictured in Figure 4.39. In (*a*) a monomeric enzyme is shown, and in panel (*b*) an oligomeric enzyme consisting of two protomers is visualized. In both models heterotropic interactions can occur between the activator and substrate sites. In model (*b*), homotropic interactions can occur between the activator sites or between the substrate sites.

Allosteric Enzymes Exhibit Sigmoidal Kinetics

As a consequence of interaction between substrate site, activator site, and inhibitor site, a characteristic sigmoid or **S**-shaped curve is obtained in [S] versus v_0 plots of allosteric enzymes, as shown in Figure 4.40 (curve A). Negative allosteric effectors move the curve toward higher substrate concentrations and enhance the sigmoidicity of the curve. If we use $1/2v_{max}$ as a guideline, Figure 4.40 shows that a higher concentration of substrate would be required to achieve $1/2v_{max}$ in the presence of a negative effector (curve C) than required in the absence

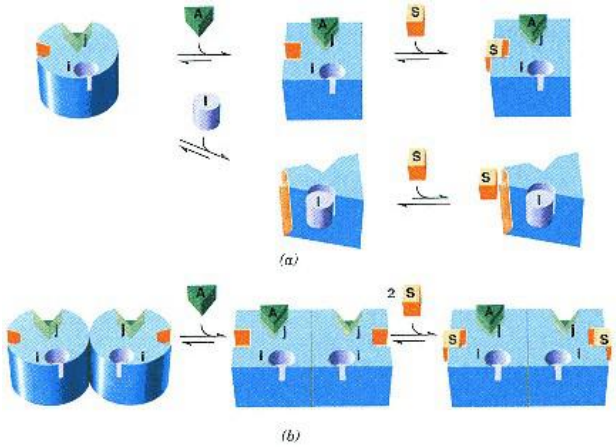


Figure 4.39
Models of allosteric enzyme systems.
(a) Model of a monomeric enzyme. Binding of a positive allosteric effector, A (green), to the activator site, j, induces a new conformation to the enzyme, one that has a greater affinity for the substrate. Binding of a negative allosteric effector (purple) to the inhibitor site, i, results in an enzyme conformation having a decreased affinity for substrate (orange).
(b) A model of a polymeric allosteric enzyme. Binding of the positive allosteric effector, A, at the j site causes an allosteric change in the conformation of the protomer to which the effector binds. This change in the conformation is transmitted to the second protomer through cooperative protomer–protomer interactions. The affinity for the substrate is increased in both protomers. A negative effector decreases the affinity for substrate of both protomers.

of negative effector (curve A). In the presence of a positive modulator (curve B), $1/2v_{max}$ can be reached at a lower substrate concentration than is required in the absence of the positive modulator (curve A). Positive modulators shift the v_0 versus [S] plots toward the hyperbolic plots observed in Michaelis–Menten kinetics.

The rates of allosteric-controlled enzymes can be finely controlled by small fluctuations in the level of substrate; often the *in vivo* concentration of substrate corresponds with the sharply rising segment of the sigmoid v_0 versus [S] plot; thus large changes in enzyme activity are effected by small changes in substrate concentration (see Figure 4.40). It is also possible to "turn an enzyme off" with small amounts of a negative allosteric effector by having the apparent K_m shifted to values well above the *in vivo* level of substrate. Note that at a given *in vivo* concentration of substrate the initial velocity, v_0, is decreased in the presence of a negative effector (compare curves A and C).

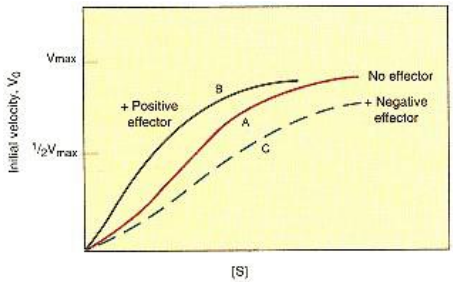


Figure 4.40
Kinetic profile of a K class allosteric enzyme.
The enzyme shows sigmoid V_0 versus [S] plots. Negative effectors shift the curve to the right, resulting in an increase in K_m. Positive effectors shift the curve to the left and effectively lower the apparent K_m. The V_{max} is not changed.

Cooperativity Explains Interaction between Ligand Sites in an Oligomeric Protein

Since allosteric enzymes are usually oligomeric with sigmoid [S] versus v_0 plots, the concept of cooperativity was proposed to explain the interaction between ligand sites in oligomeric enzymes. **Cooperativity** is the influence that the binding of a ligand to one protomer has on the binding of ligand to another protomer in an oligomeric protein. It should be emphasized that kinetic mechanisms other than cooperativity can also produce sigmoid v_0 versus [S] plots; consequently, sigmoidicity is not diagnostic of cooperativity in a v_0 versus [S] plot. The relationship between allosterism and cooperativity has frequently been confused. Conformational change occurring in a given protomer in response to ligand binding at an allosteric site is an allosteric effect. Cooperativity generally involves a change in conformation of an effector-bound protomer that in turn transforms an adjacent protomer into a new conformation with an altered affinity for the effector ligand or for a second ligand. The conformation change may be induced by an allosteric effector or it may be induced by substrate, as it is in the case of hemoglobin where the oxygen-binding site on each protomer corresponds to the substrate site on an enzyme rather than to an allosteric site. Therefore the oxygen-induced conformational change in the hemoglobin protomers is technically not an allosteric effect, although some authors describe it as such. It is a homotropic cooperative interaction. Those who consider the oxygen-induced changes in hemoglobin to be "allosteric" are using the term in a much broader sense than the original definition allows; however, "allosteric" is now used by many to describe any ligand-induced change in the tertiary structure of a protomer.

An allosteric effect can occur in the absence of any cooperativity. For example, in **alcohol dehydrogenase,** conformational changes occur independently in each of the protomers upon the addition of positive allosteric effectors. The active site of each protomer is completely independent of the other and there is no cooperativity between protomers; that is, induced conformational changes in one protomer are not transmitted to adjacent protomers.

To describe experimentally observed ligand saturation curves mathematically, several models of cooperativity have been proposed. The two most prominent are the concerted model and the sequential induced-fit model. Although the **concerted model** is rather restrictive, most of the nomenclature associated with allosterism and cooperativity arose from it. The model proposes that the enzyme exists in only two states, the T (tense or taut) and the R (relaxed) (Figure 4.41*a*). The T and R states are in equilibrium. Activators and substrates favor the R state and shift the preexisting equilibrium toward the R state by the law of mass action. Inhibitors favor the T state. A conformational change in one protomer causes a corresponding change in all protomers. No hybrid states occur. Although this model accounts for the kinetic behavior of many enzymes, it cannot account for negative cooperativity.

The **sequential induced-fit model** proposes that ligand binding induces a conformational change in a protomer. A corresponding conformational change is then partially induced in an adjacent protomer contiguous with the protomer containing the bound ligand. The effect of ligand binding is sequentially transmitted through the oligomer, producing increased or decreased affinity for the ligand by contiguous protomers (Figure 4.41 *b*). In this model numerous hybrid states occur, giving rise to cooperativity and sigmoid [S] versus v_0 plots. Both positive and negative cooperativity can be accommodated by the model. A positive modulator induces a conformation in the protomer, which has an increased affinity for the substrate. A negative modulator induces a different conformation in the protomer, one that has a decreased affinity for substrate. Both effects are cooperatively transmitted to adjacent protomers. For the V class enzymes the effect is on the catalytic event (k_3) rather than on K_m.

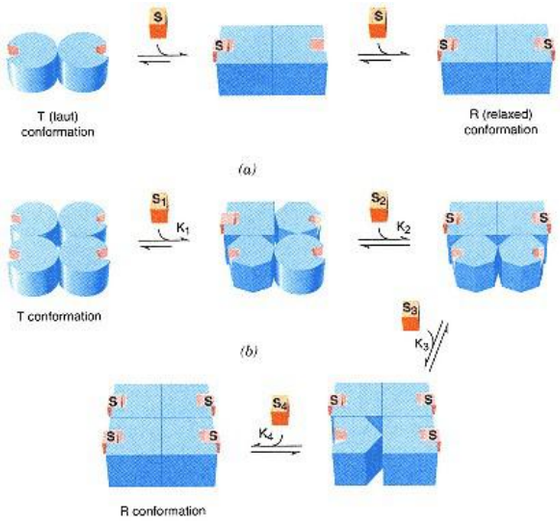


Figure 4.41
Models of cooperativity.
(a) The concerted model. The enzyme exists in only two states, the T (tense or taut) and R (relaxed) conformations. Substrates and activators have a greater affinity for the R state and inhibitors for the T state. Ligands shift the equilibrium between the T and R states.
(b) The sequential induced-fit model. Binding of a ligand to any one subunit induces a conformational change in that subunit. This conformational change is transmitted partially to adjoining subunits through subunit–subunit interaction. Thus the effect of the first ligand bound is transmitted cooperatively and sequentially to the other subunits (protomers) in the oligomer, resulting in a sequential increase or decrease in ligand affinity of the other protomers. The cooperativity may be either positive or negative, depending on the ligand.

Regulatory Subunits Modulate the Activity of Catalytic Subunits

In the foregoing an allosteric site was considered to reside on the same protomer as the catalytic site and all protomers were considered to be identical. In several very important enzymes a distinct regulatory protomer exists. These **regulatory subunits** have no catalytic function, but their binding with the catalytic protomer modulates the activity of the catalytic subunit through an induced conformational change. One strategy for regulation by regulatory subunits is outlined in Figure 4.42 for the **protein kinase A (PKA)** complex. Each regulatory subunit (R) has a segment of its primary sequence that is a pseudosubstrate for the catalytic subunit (C). In the absence of cAMP, the R subunit binds to the C subunit at its active site through the pseudosubstrate sequence, which inhibits the protein kinase activity. When cellular cAMP levels rise, cAMP binds to a site on the R subunits, causing a conformational change. This removes the pseudosubstrate sequence from the active site of the C subunit. The C subunits are released and can accept other protein substrates containing the pseudosubstrate sequence.

Calmodulin, a 17-kDa Ca^{2+}-binding protein, is a regulatory subunit for enzymes using Ca^{2+} as a modulator of their activity. Binding of calcium to calmodulin induces a conformational change in calmodulin allowing it to bind to the Ca^{2+}-dependent enzyme. This binding induces a conformational change in the enzyme, restoring enzymatic activity.

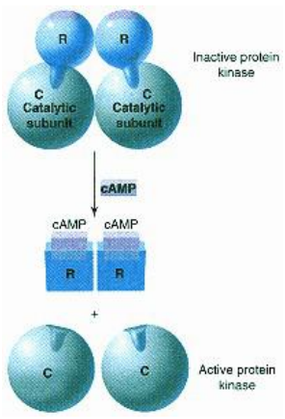


Figure 4.42
Model of allosteric enzyme with separate catalytic (C) and regulatory (R) subunits.
The regulatory subunit of protein kinase A contains a pseudosubstrate region in its primary sequence that binds to the substrate site of the catalytic subunit. In the presence of cAMP the conformation of the R subunit changes so that the pseudosubstrate region can no longer bind, resulting in release of active C subunits.

4.7— Enzyme Specificity: The Active Site

Enzymes are the most specific catalysts known, as regards the substrate and the type of reaction undergone by substrate. Specificity resides in the **substrate-binding site** on the enzyme surface. The tertiary structure of the enzyme is folded in such a way as to create a region that has the correct molecular dimensions, the appropriate topology, and the optimal alignment of counter-

ionic groups and hydrophobic regions to accommodate a specific substrate. The tolerances in the active site are so small that usually only one isomer of a diastereomeric pair will bind. For example, D-amino acid oxidase will bind only D-amino acids but not L-amino acids. Some enzymes show absolute specificity for substrate. Others have broader specificity and will accept several different analogs of a specific substrate. For example, hexokinase catalyzes the phosphorylation of glucose, mannose, fructose, glucosamine, and 2-deoxyglucose, but at different rates. Glucokinase, on the other hand, is specific for glucose.

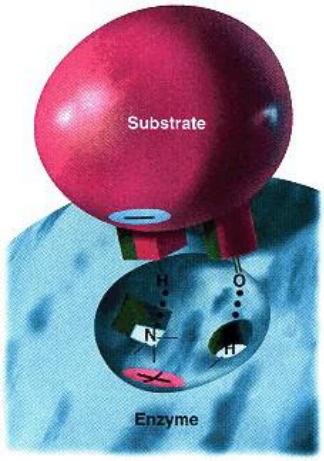


Figure 4.43
Lock-and-key model of the enzyme-binding site.
The enzyme contains a negative impression of the molecular features of the substrate, thus allowing specificity of the enzyme for a particular substrate. Specificat ion pair formation can contribute to recognition of the substrate.

The specificity of the reaction rests in the active site and the amino acids that participate in the bond-making and bond-breaking phase of catalysis (see Section 4.8).

Complementarity of Substrate and Enzyme Explains Substrate Specificity

Various models have been proposed to explain the substrate specificity of enzymes. The first proposal was the **"lock-and-key" model** (Figure 4.43), in which a negative impression of the substrate is considered to exist on the enzyme surface. Substrate fits in this binding site just as a key fits into the proper lock or a hand into the proper sized glove. Hydrogen and ionic bonding and hydrophobic interactions contribute in binding substrate to the binding site. This model gives a rigid picture of the enzyme and cannot account for the effects of allosteric ligands.

A more flexible model of the binding site is provided by the **induced fit model** in which the binding and active sites are not fully preformed. The essential elements of the binding site are present to the extent that the correct substrate can position itself properly. Interaction of substrate with enzyme induces a conformational change in the enzyme, resulting in the formation of a stronger binding site and the repositioning of the appropriate amino acids to form the active site. There is excellent X-ray evidence for this model with carboxypeptidase A. A schematic of the induced-fit model is shown in Figure 4.44*a*. Figure 4.45 shows a significant movement of the lower lobe of **hexokinase** on binding glucose. The hexokinase essentially closes around the glucose to bring the active-site residues into proximity with the glucose.

Induced fit combined with **substrate strain** accounts for more experimental observations concerning enzyme action than other models. In this model (Figure 4.44*b*), substrate is "strained" toward product formation by an induced conformational transition of the enzyme. A good example of enzyme-induced

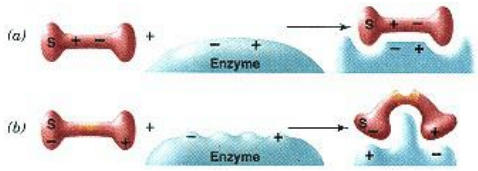


Figure 4.44
Models for induced fit and substrate strain.
(a) Approach of substrate to the enzyme induces the formation of the active site.
(b) Substrate strain, induced by substrate binding to the enzyme, contorts normal bond angles and "activates" the substrate.
Reprinted with permission from Koshland, D. *Annu. Rev. Biochem.* 37:374, 1968.

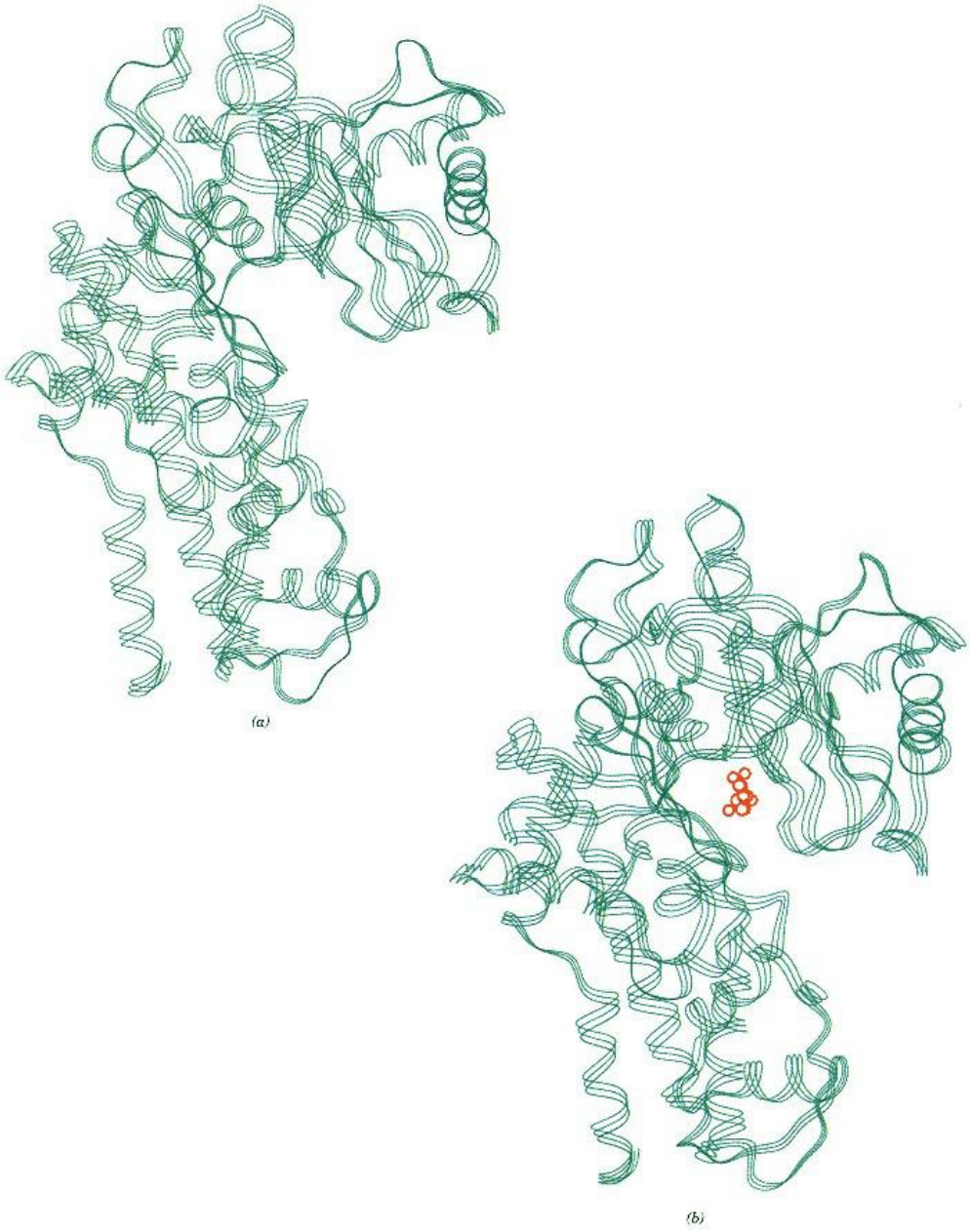


Figure 4.45
Glucose induced conformational change of hexokinase.
(a) Hexokinase minus glucose.
(b) Hexokinase with glucose. The three-cord ribbon traces the peptide backbone of hexokinase.
Drawn from PDB files 1HKG and 2YHX; Bennett, W. S. Jr., and Steitz, T. A. *J. Mol. Biol.* 140: 211, 1980.

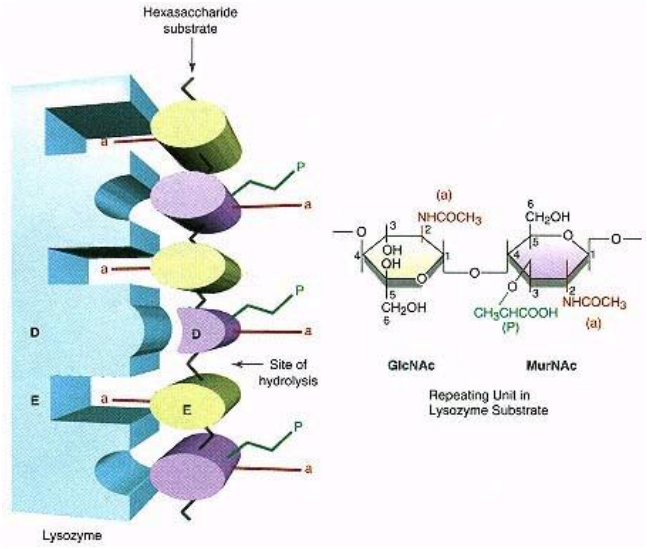


Figure 4.46
Hexasaccharide binding at active site of lysozyme. In the model substrate pictured, the ovals represent individual pyranose rings of the repeating units of the lysozyme substrate shown to the right. Ring D is strained by the enzyme to the half-chair conformation and hydrolysis occurs between the D and E rings. Six subsites on the enzyme bind substrate. Alternate sites are specific for acet-amido groups
(a) but are unable to accept the lactyl (P) side chains, which occur on the N-acetylmuramic acid residues. Thus the substrate can bind to the enzyme in only one orientation.
Redrawn based on model proposed by Imoto, T., et al. In P. Boyer (Ed.), *The Enzymes*, 3rd ed., Vol. 7. New York: Academic Press, 1972, p. 713.

substrate strain is that of **lysozyme** (Figure 4.46) in which the conformation of the sugar residue "D" at which bond breaking occurs is strained from the stable chair to the unstable half-chair conformation upon binding. These conformations of glucose are shown in Figure 4.47. The concept of substrate strain explains the role of the enzyme in increasing the rate of reaction (see Section 4.8).

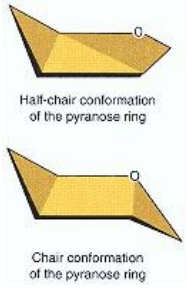
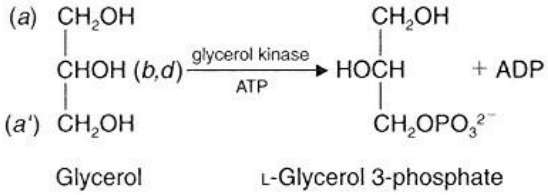


Figure 4.47
Two possible conformations of glucose.

Asymmetry of the Binding Site

Not only are enzymes able to distinguish between isomers of the substrate, but they are able to distinguish between two equivalent atoms in a symmetrical molecule. For example, glycerol kinase distinguishes between configurations of H and OH on C-2 in the symmetric substrate glycerol, so that only the asymmetric product L-glycerol 3-phosphate is formed. These **prochiral substrates** have two identical substituents and two additional but dissimilar groups on the same carbon ($C_{aa'bd}$).



Prochiral substrates possess no optical activity but can be converted to chiral compounds, that is, ones that possess an asymmetric center. The explanation for this enigma is provided if the enzyme binds the two dissimilar groups at specific sites and only one of the two similar substituents is able to bind at the active site (Figure 4.48). Thus the enzyme is able to recognize only one specific orientation of the symmetrical molecule. Asymmetry is produced in the product by modification of one side of the bound substrate. A minimum of three different binding sites on the enzyme surface is required to distinguish between identical groups on a prochiral substrate.

4.8—
Mechanism of Catalysis

All chemical reactions have a potential energy barrier that must be overcome before reactants can be converted to products. In the gas phase the reactant molecules can be given enough kinetic energy by heating them so that collisions result in product formation. The same is true with solutions. However, a well-controlled body temperature of 37°C does not allow temperature to be increased to accelerate the reaction, and 37°C is not warm enough to provide the reaction rates required for fast-moving species of animals. Enzymes employ other means of overcoming the barrier to reaction.

Diagrams for catalyzed and noncatalyzed reactions are shown in Figure 4.49. The energy barrier represented by the uncatalyzed curve in Figure 4.49 is a measure of the **activation energy**, E_a, required for the reaction to occur. The reaction coordinate is simply the pathway in terms of bond stretching between reactants and products. At the apex of the energy barrier is the activated complex known as the **transition state**, Ts, that represents the reactants in their activated state. In this state reactants are in an intermediate stage along the reaction pathway and cannot be identified as starting material or products. For example, in the hydrolysis of ethyl acetate:

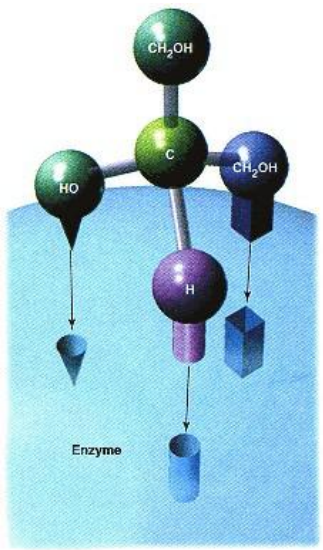
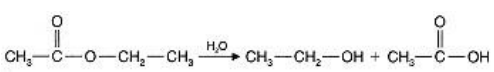
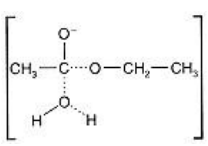


Figure 4.48
Three-point attachment of a symmetrical substrate to an asymmetric substrate-binding site.
Glycerol kinase by virtue of dissimilar binding sites for the –H and –OH group of glycerol binds only the -hydroxymethyl group to the active site. One stereoisomer results from the kinase reaction, L-glycerol 3-phosphate.



the Ts might look like



The transition state complex can break down to products or go back to reactants. The Ts is not an intermediate and cannot be isolated! In the case of the enzyme-catalyzed reaction (Figure 4.49) the energy of the reactants and products is no different than in the uncatalyzed reaction. Enzymes do not change the thermodynamics of the system but they do change the pathway for reaching the final state.

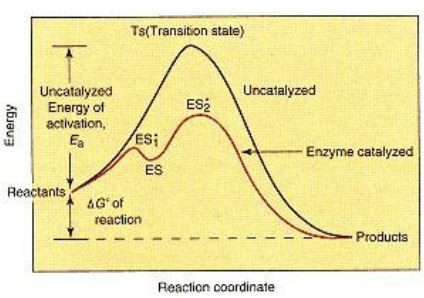


Figure 4.49
Energy diagrams for catalyzed versus noncatalyzed reactions.
The overall energy difference between reactants and products is the same in catalyzed and noncatalyzed reactions. The enzyme-catalyzed reaction proceeds at a faster rate because the energy of activation is lowered.

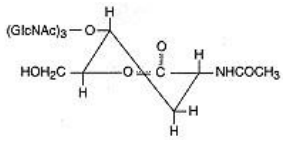


Figure 4.50
A transition state analog (tetra-*N*-acetyl-chitotetrose-*d*-lactone) of ring D of the substrate for lysozyme.

As noted on the energy diagram, there may be several plateaus or valleys on the energy contour for an enzyme reaction. At these points metastable intermediates exist. An important point is that each valley may be reached with the heat input available in 37°C. The enzyme allows the energy barrier to be scaled in increments. The Michaelis–Menten ES complex is not the transition state but may be found in one of the valleys because in the ES complex substrates are properly oriented and may be "strained." The bonds to be broken lie further along the reaction coordinate.

If our concepts of the transition state are correct, one would expect that compounds designed to resemble closely the transition state would bind more tightly to the enzyme than the natural substrate. This has proved to be the case. In such substrate analogs one finds affinities 10^2–10^5 times greater than those for substrate. These compounds are called **transition state analogs** and are potent enzyme inhibitors. Previously, lysozyme was discussed in terms of substrate strain, and mention was made of the conversion of sugar ring D from a chair to a strained half-chair conformation. Synthesis of a transition state analog in the form of the δ-lactone of tetra-*N*-acetylchitotetrose (Figure 4.50), which has a distorted half-chair conformation, followed by binding studies, showed that this transition state analog was bound 6000 times tighter than the normal substrate.

Enzymes Decrease Activation Energy

Enzymes can enhance the rates of reaction by a factor of 10^9–10^{12} times that of the noncatalyzed reaction. Most of this rate enhancement can be accounted for by four processes: acid–base catalysis, substrate strain (transition state stabilization), covalent catalysis, and entropy effects.

Acid–Base Catalysis

Specific acids and bases are H^+ and OH^-, respectively. Free protons and hydroxide ions are not encountered in most enzyme reactions and then only in some metal-dependent enzymes (see p. 144). A **general acid** or base is a compound that is weakly ionizable. In the physiological pH range, the protonated form of histidine is the most important general acid and its conjugate base is an important general base (Figure 4.51). Other acids are the thiol –SH of cysteine, tyrosine –OH, and the ε-amino group of lysine. Other bases are carboxylic acid anions and the conjugate bases of the general acids.

Ribonuclease (RNase) exemplifies the role of acid and base catalysis at the enzyme active site. RNase cleaves an RNA chain at the 3 -phosphodiester linkage of pyrimidine nucleotides with an obligatory formation of a cyclic 2 , 3 -phosphoribose on a pyrimidine nucleotide as intermediate. In the mechanism outlined in Figure 4.52, His 119 acts as a general acid to protonate the phosphodiester bridge, whereas His 12 acts as a base in generating an alkoxide on the ribose-3 -hydroxyl. The latter then attacks the phosphate group, forming a cyclic phosphate and breakage of the RNA chain at this locus. The cyclic phosphate is then cleaved in phase 2 by a reversal of the reactions in phase 1, but with water replacing the leaving group. The active-site histidines revert to their original protonated state.

Substrate Strain

Previous discussion of this topic related to induced fit of enzymes to substrate. Binding of substrate to a preformed site on the enzyme can induce strain in the substrate. Irrespective of the mechanism of strain induction, the energy level of the substrate is raised, and the bond lengths and angles of the substrate more closly resemble those found in the transition state.

A combination of substrate strain and acid–base catalysis is observed in the action of lysozyme (Figure 4.53). X-ray evidence shows that ring D of the

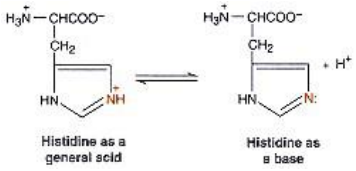


Figure 4.51
Acid and base forms of histidine.

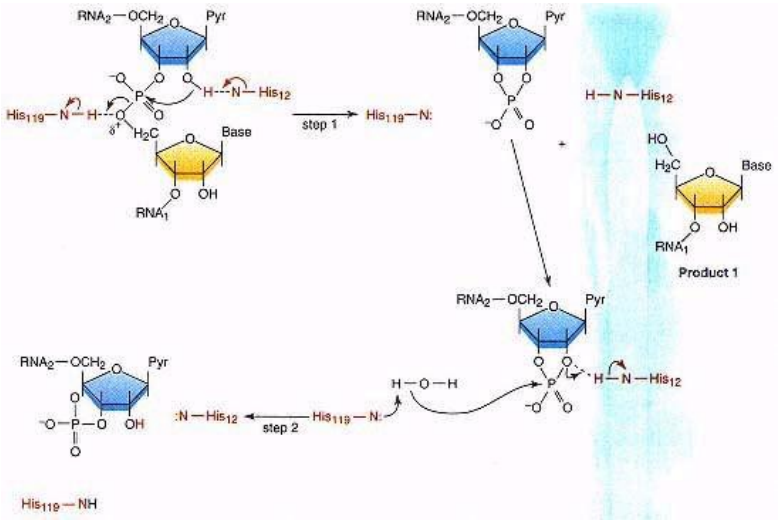


Figure 4.52
Role of acid and base catalysis in the active site of ribonuclease.
RNase cleaves the phosphodiester bond in a pyrimidine locus in RNA. Histidine residues 12 and 119, respectively, at the ribonuclease active site function as acid and base catalysts in enhancing the formation of an intermediate 2 , *3* -cyclic phosphate and release of a shorter fragment of RNA (product 1). These same histidines then play a reverse role in the hydrolysis of the cyclic phosphate and release of the other fragment of RNA (product 2) that ends in a pyrimidine nucleoside *3* -phosphate. As a result of the formation of product 2, the active site of the enzyme is regenerated.

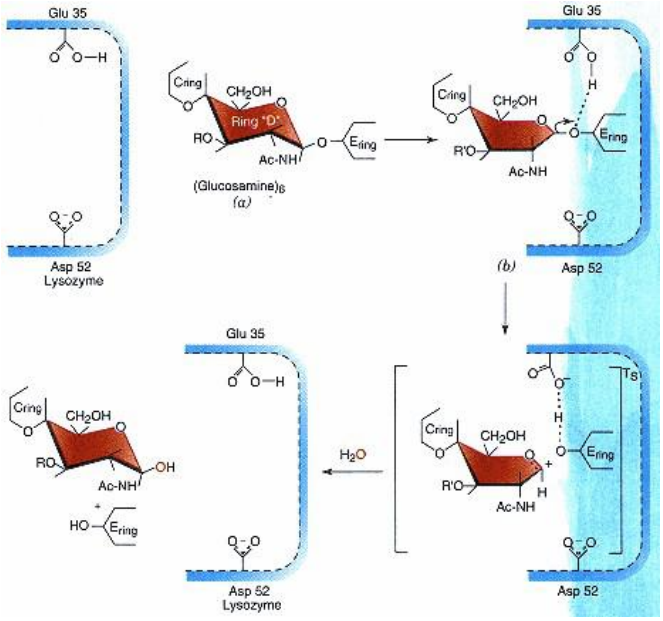


Figure 4.53
Mechanism for lysozyme action: substrate strain.
Binding of the stable chair
(a) conformation of the substrate to the enzyme generates the strained half-chair conformation
(b) in the ES complex. In the transition state, acid-catalyzed hydrolysis of the glycosidic linkage by an active-site glutamic acid residue generates a carbonium ion on the D ring, which relieves the strain generated in the initial ES complex and results in collapse of the transition state to products.

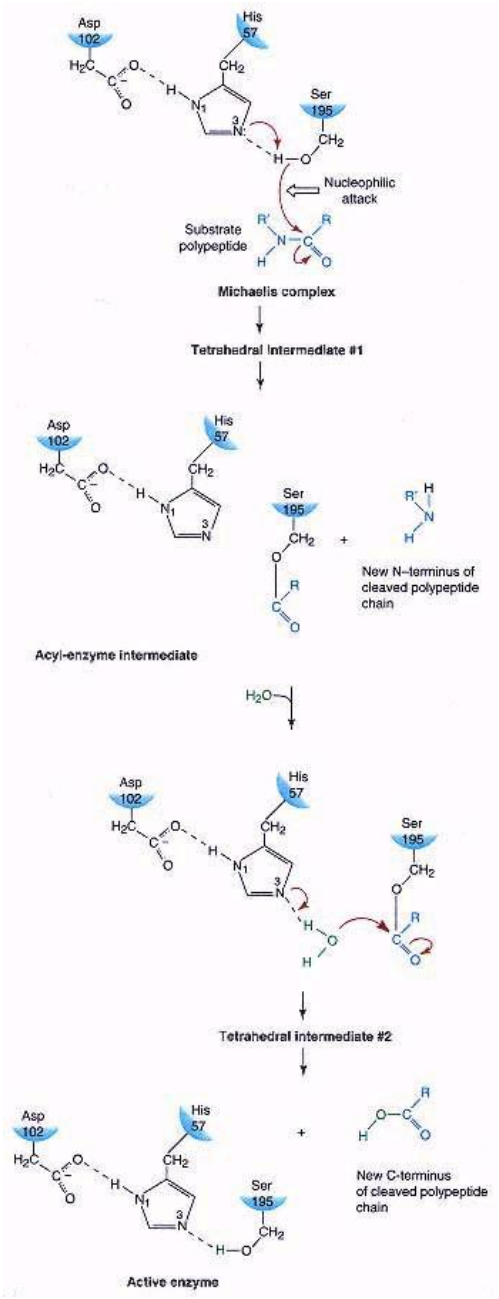


Figure 4.54
Covalent catalysis in active site of chymotrypsin.
Through acid-catalyzed nucleophilic attack, as shown by red arrows, the stable amide linkage of the peptide substrate is converted into an unstable acylated enzyme through serine-195 of the enzyme. The latter is hydrolyzed in the rate-determining step. The new amino-terminal peptide, shown in blue, is released concomitant with formation of the acylated enzyme.

hexasaccharide substrate is strained to the half-chair conformation upon binding to lysozyme. General acid catalysis by active-site glutamic acid promotes the unstable half-chair into the transition state. The oxycarbonium ion formed in the transition state is stabilized by the negatively charged aspartate. Breakage of the glycosidic linkage between rings D and E relieves the strained transition state by allowing ring D to return to the stable chair conformation.

Covalent Catalysis

In **covalent catalysis,** the attack of a nucleophilic (negatively charged) or electrophilic (positively charged) group in the enzyme active site upon the substrate results in covalent binding of the substrate to the enzyme as an intermediate in the reaction sequence. Enzyme-bound coenzymes often form covalent bonds with the substrate. For example, in the **transaminases,** the amino acid substrate forms a Schiff base with enzyme-bound pyridoxal phosphate (see p. 449). In all cases of covalent catalysis, the enzyme- or coenzyme-bound substrate is more labile than the original substrate. The enzyme–substrate adduct represents one of the valleys on the energy profile.

Serine proteases, such as trypsin, chymotrypsin, and thrombin, are good representatives of the covalent catalytic mechanism (see p. 97). Acylated enzyme has been isolated in the case of chymotrypsin. Covalent catalysis is assisted by acid–base catalysis in these particular enzymes (Figure 4.54). In chymotrypsin the attacking nucleophile is Ser 195, which is not dissociated at pH 7.4 and a mechanism for ionizing this very basic group is required. It is now thought that in the anhydrous milieu of the active site, Ser 195 and His 57 have similar p*K* values and that the negative charge on Asp 102 stabilizes the transfer of the proton from the OH of Ser 195 to N3 of His 57 (Figure 4.54). The resulting serine alkoxide attacks the carbonyl carbon of the peptide bond, releasing the amino-terminal end of the protein and forming an **acylated enzyme** intermediate (through Ser 195). The acylated enzyme is then cleaved by reversal of the reaction sequence, but with water as the nucleophile rather than Ser 195. Chemical evidence indicates the formation of two tetrahedral intermediates, one preceding the formation of the acylated enzyme and one following the attack of water on the acyl-enzyme (Figure 4.55).

Transition State Stabilization

The previously mentioned effects promote the substrate to enter the transition state. Since the active site binds the transition state with a much greater affinity than the substrate, that small fraction of substrate molecules existing in a transition state geometry will be converted to products quickly. Thus, by mass action, all the substrate can be rapidly converted to products. Any factor that increases the population of substrate molecules resembling the transition state will contribute to catalysis.

Entropy Effect

Entropy is a thermodynamic term, S, which defines the extent of disorder in a system. At equilibrium, entropy is maximal. For example, in solution two reactants A and B exist in many different orientations. The chances of A and B coming together with the correct geometric orientation and with enough energy to react is small at 37°C and in dilute solution. However, if an enzyme with two high-affinity binding sites for A and B is introduced into the dilute solution of these reactants, as suggested in Figure 4.56, A and B will be bound to the enzyme in the correct orientation for the reaction to occur. They will be bound with the correct stoichiometry, and the effective concentration of the reactants will be increased on the enzyme surface, all of which will contribute to an increased rate of reaction.

When correctly positioned and bound on the enzyme surface, the substrates may be "strained" toward the transition state. At this point the substrates have

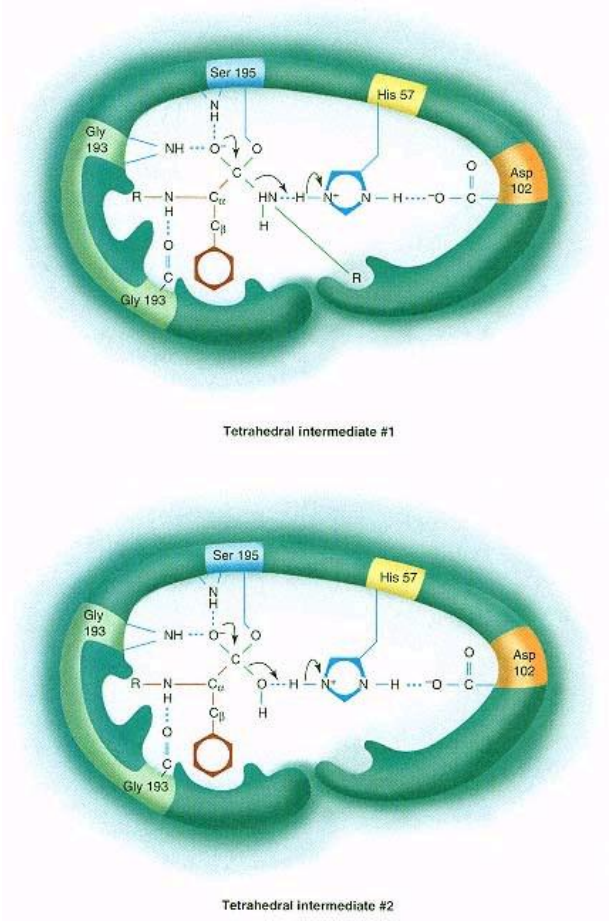


Figure 4.55
Tetrahedral intermediates.
(a) Model of tetrahedral intermediate #1 that precedes formation of the acyl-enzyme intermediate.
(b) Model of tetrahedral intermediate #2 resulting from the attack of water on acyl-enzyme intermediate.

been "set up" for acid–base and/or covalent catalysis. Proper orientation and the nearness of the substrate with respect to the catalytic groups, which has been dubbed the "proximity effect," contribute 10^3–10^5-fold to the rate enhancement observed with enzymes. It is estimated that the decrease in entropy contributes a factor of 10^3 to the rate enhancement.

Abzymes Are Artificially Synthesized Antibodies with Catalytic Activity

If the principles discussed above for enzyme catalysis are correct, then one should be able to design an artificial enzyme. This feat has been accomplished by the use of several different approaches, but only the synthesis of antibodies that have catalytic activity will be considered in this discussion. These antibodies are called **abzymes.** Design of abzymes is based on two principles. The first principle is the ability of the immune system to recognize any arrangement

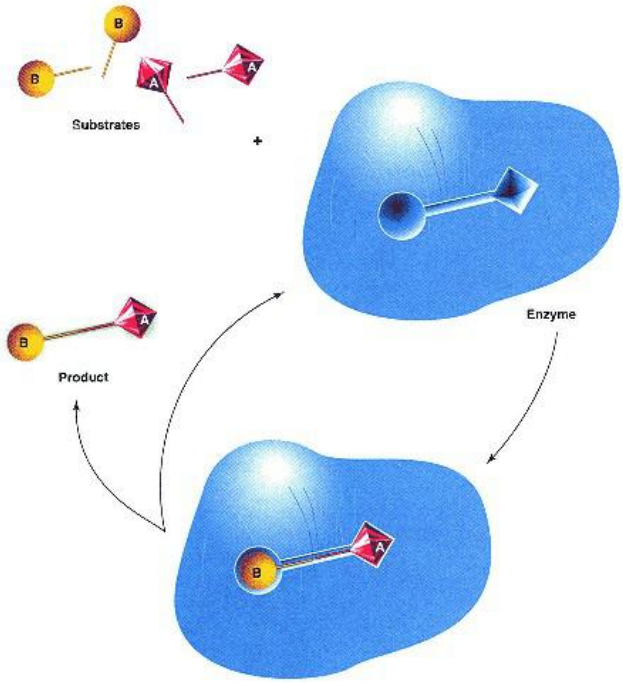


Figure 4.56
Role of the enzyme in enhancing reaction rate by decreasing entropy.
Substrates in dilute solution are concentrated and oriented on the enzyme surface so as to enhance the rate of the reaction.

of atoms in the foreign antigen and to make a binding site on the resulting immunoglobulin that is exquisitely suited to binding that antigen. The second principle is that strong binding of transition state-like substrates reduces the energy barrier along the reaction pathway (see discussion on p. 160).

In abzymes a transition state analog serves as the hapten. For a lipase abzyme, a racemic phosphonate (Figure 4.57) serves as a hapten. Two enantiomeric fatty acid ester substrates are shown in Figure 4.57*b,c*. See page 159 for the transition state structure expected for ester hydrolysis. Among many antibodies produced by rabbits on challenge with the protein-bound transition state analog (Figure 4.57*a*), one hydrolyzed only the (*R*) isomer (Figure 4.57*b*) and another only the (*S*) isomer. These abzymes enhanced the rate of hydrolysis of substrates (*a*) and (*b*) 10^3–10^5-fold above the background rate in a stereospecific manner. Acceleration of 10^6-fold, which is close to the enzymatic rate, has been achieved in another esterase-like system.

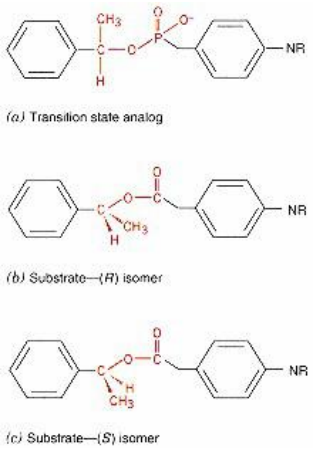


Figure 4.57
Hapten and substrate for a catalytic antibody (abzyme). Phosphonate (*a*) is the transition state analog used as the hapten to generate antibodies with lipase-like catalytic activity. Specific abzymes can be generated for either the(*R*) isomer (*b*) or the (*S*) isomer (*c*) of methyl benzyl esters.

Environmental Parameters Influence Catalytic Activity

A number of external parameters, including pH, temperature, and salt concentration, affect enzyme activity. These effects are probably not important *in vivo* under normal conditions but are very important in setting up enzyme assays *in vitro* to measure enzyme activity in samples of a patient's plasma or tissue.

Temperature

Plots of velocity versus temperature for most enzymes reveal a bell-shaped curve with an optimum between 40°C and 45°C for mammalian enzymes, as indicated in Figure 4.58. Above this temperature, heat denaturation of the enzyme occurs. Between 0°C and 40°C, most enzymes show a twofold increase in activity for every 10° C rise. Under conditions of hypothermia, most enzyme reactions are depressed, which accounts for the decreased oxygen demand of living organisms at low temperature. Mutation of an enzyme to a thermolabile form can have serious consequences (see Clin. Corr. 4.5).

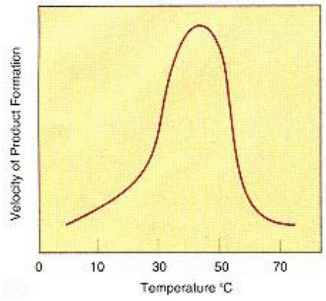


Figure 4.58
Temperature dependence of a typical mammalian enzyme.
To the left of the optimum, the rate is low because the environmental temperature is too low to provide enough kinetic energy to overcome the energy of activation. To the right of the optimum, the enzyme is inactivated by heat denaturation.

pH

Nearly all enzymes show a bell-shaped pH–velocity profile, but the maximum **(pH optimum)** varies greatly with different enzymes. Alkaline and acid phosphatases with very different pH optima are both found in humans, as shown in Figure 4.59. The bell-shaped curve and its position on the x-axis are dependent on the particular ionized state of the substrate that will be optimally bound to the enzyme. This in turn is related to the ionization of specific amino acid residues that constitute the substrate-binding site. In addition, amino acid residues involved in catalyzing the reaction must be in the correct charge state to be functional. For example, if aspartic acid is involved in catalyzing the reaction, the pH optimum may be in the region of 4.5 at which the α-carboxyl of aspartate ionizes; whereas if the ε-amino of lysine is the catalytic group, the pH optimum may be around 9.5, the pK_a of the ε-amino group. Studies of the pH dependence of enzymes are useful for suggesting which amino acid(s) may be operative in catalysis.

Clinical Correlation 4.6 points out the effect of a mutation leading to a change in the pH optimum of a physiologically important enzyme. Such a mutated enzyme may function on the shoulder of the pH-rate profile, but not be optimally active, even under normal physiological conditions. When an abnormal condition such as alkalosis (observed in vomiting) or acidosis (observed in pneumonia and often in surgery) occurs, the enzyme activity may disappear because the pH is inappropriate. Thus under normal conditions, the enzyme may be active enough to meet normal requirements, but under stress conditions the enzyme may be less active.

CLINICAL CORRELATION 4.5

Thermal Lability of Glucose-6-Phosphate Dehydrogenase Results in Hemolytic Anemia

In red cells, glucose-6-phosphate (G6PD) is an important enzyme in the red cell for the maintenance of the membrane integrity. A deficiency or inactivity of this enzyme leads to a hemolytic anemia. In other cases, a variant enzyme is present that normally has sufficient activity to maintain the membrane but fails under conditions of oxidative stress. A mutation of this enzyme leads to a protein with normal kinetic constants but a decreased thermal stability. This condition is especially critical to the red cell, since it is devoid of protein-synthesizing capacity and cannot renew enzymes as they denature. The end result is a greatly decreased lifetime for those red cells that have an unstable G6PD. These red cells are also susceptible to drug-induced hemolysis. See Clin. Corr. 8.1.

Lazzatio, L., and Meta, A., Glucose-6-phosphate dehydrogenase deficiency. In: C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed. New York: McGraw-Hill, 1995, p. 3369.

4.9— Clinical Applications of Enzymes

The principles of enzymology outlined in previous sections are applied in the clinical laboratory in measurement of plasma or tissue enzyme activities and concentrations of substrates in patients. The rationale for measuring plasma enzyme activities is based on the premise that changes in activities reflect changes that have occurred in a specific tissue or organ. Plasma enzymes are of two types: (1) one type is present in the highest concentration, is specific to plasma, and has a functional role in plasma; and (2) the second is normally present at very low levels and plays no functional role in the plasma. The former includes the enzymes associated with blood coagulation (e.g., thrombin), fibrin dissolution (plasmin), and processing of chylomicrons (lipoprotein lipase).

In disease of tissues and organs, the nonplasma-specific enzymes are most important. Normally, the plasma levels of these enzymes are low to absent. A disease process may cause changes in cell membrane permeability or increased cell death, resulting in release of intracellular enzymes into the plasma. When permeability changes, those enzymes of lower molecular weight will appear in the plasma first and the greater the concentration gradient between intra- and extracellular levels, the more rapidly the enzyme diffuses out. Cytosolic enzymes will appear in the plasma before mitochondrial enzymes, and the

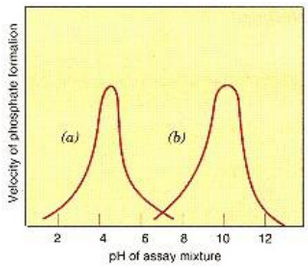


Figure 4.59
The pH dependence of
(*a*) acid and
(*b*) alkaline phosphatase reactions.
In each case the optimum represents the ideal ionic state for binding of enzyme and substrate and the correct ionic state for the amino acids involved in the catalytic event.

greater the quantity of tissue damaged, the larger the increase in plasma level. The nonplasma-specific enzymes will be cleared from the plasma at varying rates, which depend on the stability of the enzyme and its uptake by the reticuloendothelial system.

CLINICAL CORRELATION 4.6

Alcohol Dehydrogenase Isoenzymes with Different pH Optima

In addition to the change in aldehyde dehydrogenase isoenzyme composition in some Asians (see Clin. Corr. 4.2), different alcohol dehydrogenase isoenzymes are also observed. Alcohol dehydrogenase (ADH) is encoded by three genes, which produce three different polypeptides: α, β, and γ. Three alleles are found for the β-gene that differ in a single nucleotide base, which causes substitutions for arginine. The substitutions are shown below:

	Residue 47	*Residue 369*
β_1	Arg	Arg
β_2	His	Arg
β_3	Arg	Cys

The liver β_3 form has ADH activity with a pH optimum near 7, compared with 10 for β_1, and 8.5 for β_2. The rate-determining step in alcohol dehydrogenase is the release of NADH. NADH is held on the enzyme by ionic bonds between the phosphates of the coenzyme and the arginines at positions 47 and 369. In the β_1 isozyme this ionic interaction is not broken until the pH is quite alkaline and the guanidinium group of arginine starts to dissociate H^+. Substitution of amino acids with lower pK values, as in β_2 and β_3, weakens the interaction and lowers the pH optimum. Since the release of NADH is facilitated, the V_{max} values for β_2 and β_3 are also higher than for β_1.

Burnell, J. C., Carr, L. G., Dwulet, F. E., Edenberg, H. J., Li, T-K., and Bosron, W. F. The human β_3 alcohol dehydrogenase subunit differs from β_1 by a cys- for arg-369 substitution which decreased NAD(H) binding. *Biochem. Biophys. Res. Commun.* 146:1227, 1987.

In the diagnosis of specific organ involvement in a disease process it would be ideal if enzymes unique to each organ could be identified. This is unlikely because the metabolic processes of various organs are very similar. Alcohol dehydrogenase of the liver and acid phosphatase of the prostate are useful for specific identification of disease in these organs. Other than these two examples, there are few enzymes that are tissue or organ specific. However, the ratio of various enzymes does vary from tissue to tissue. This fact, combined with a study of the kinetics of appearance and disappearance of particular enzymes in plasma, allows a diagnosis of specific organ involvement to be made. Figure 4.60 illustrates the time dependence of the plasma activities of enzymes released

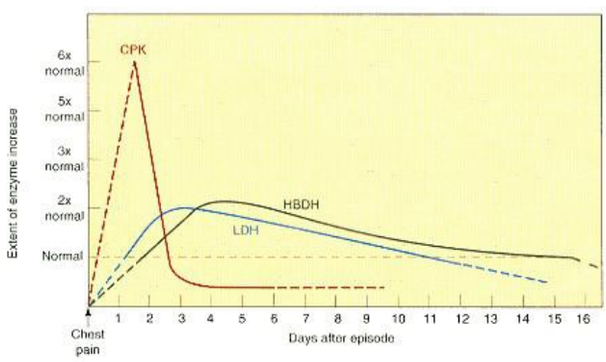


Figure 4.60
Kinetics of release of cardiac enzymes into serum following a myocardial infarction.
CPK, creatine kinase; LDH, lactic dehydrogenase; HBDH, -hydroxybutyric dehydrogenase. Such kinetic profiles allow one to determine where the patient is with respect to the infarct and recovery. Note: CPK rises sharply but briefly; HBDH rises slowly but persists.
Reprinted with permission from Coodley, E. L. *Diagnostic Enzymes*. Philadelphia: Lea & Febiger, 1970, p. 61.

from the myocardium following a heart attack. Such profiles allow one to establish when the attack occurred and whether treatment is effective. Clinical Correlation 4.7 demonstrates how diagnosis of a specific enzyme defect led to a rational clinical treatment that restored the patient to health.

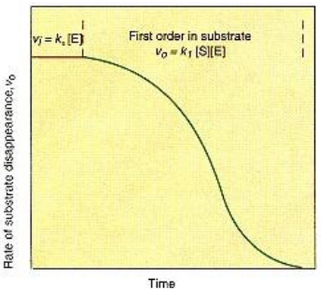


Figure 4.61
Relation of substrate concentration to order of the reaction.
When the enzyme is completely saturated, the kinetics are zero order with respect to substrate and are first order in the enzyme; that is, the rate depends only on enzyme concentration. When the substrate level falls below saturating levels, the kinetics are first order in both substrate and enzyme and are therefore second order; that is, the observed rate is dependent on both enzyme and substrate.

Studies of the kinetics of appearance and disappearance of plasma enzymes require a valid enzyme assay. A good assay is based on temperature and pH control, as well as saturating levels of all substrates, cosubstrates, and cofactors. To accomplish the latter, the K_m must be known for those particular conditions of pH, ionic strength, and so on, that are to be used in the assay. Recall that K_m is the substrate concentration at half-maximal velocity (1/2 V_{max}). To assure that the system is saturated, substrate concentration is generally increased five- to tenfold over the K_m. With saturation of the enzyme with substrate, the reaction is zero order. This fact is emphasized in Figure 4.61. Under zero-order conditions changes in velocity are proportional to enzyme concentration alone. Under first-order conditions, the velocity is dependent on both the substrate and enzyme concentrations. Clinical Correlation 4.8 demonstrates the importance of determining if the assay conditions accurately reflect the amount of enzyme actually present. Clinical laboratory assay conditions are optimized for the properties of the normal enzyme and may not correctly measure levels of mutated enzyme. pH dependence and/or the K_m for substrate and cofactors may drastically change in a mutated enzyme. Under optimal conditions a valid enzyme assay reflects a linear dependence of velocity and amount of enzyme. This can be tested by determining if the velocity of the reaction doubles when the plasma sample size is doubled, while keeping the total volume of the assay constant (Figure 4.62).

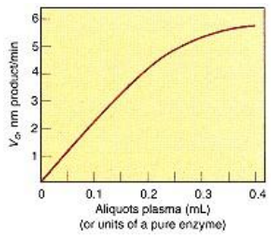


Figure 4.62
Assessing the validity of an enzyme assay.
The line shows what is to be expected for any reaction where the concentration of substrate is held constant and the aliquots of enzyme are increased. In this example linearity between initial velocity observed and amount of enzyme, whether pure or in a plasma sample, is only0160observed up to 0.2 mL of plasma or 0.2 unitsof pure enzyme. If more than 0.2 mL is used, the actual amount of enzyme in the sample would be underestimated.

Coupled Assays Utilize the Optical Properties of NAD, NADP, or FAD

Enzymes that employ the coenzymes NAD^+, $NADP^+$, and FAD are easily measured because of the optical properties of NADH, NADPH, and FAD. The absorption spectra of NADH and FAD in the ultraviolet and visible light regions are shown in Figure 4.63. Oxidized FAD absorbs strongly at 450 nm, while NADH has maximal absorption at 340 nm. The concentrations of both FAD and NADH are related to their absorption of light at the respective absorption maximum by the **Beer–Lambert relation**

$$A = \varepsilon \cdot c \cdot l$$

where l is the pathlength of the spectrophotometer cell in centimeters (usually 1 cm), ε is absorbance of a molar solution of the substance being measured at

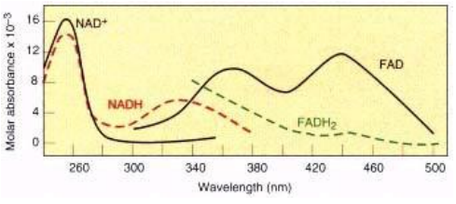


Figure 4.63
Absorption spectra of niacin and flavin coenzymes.
The reduced form of NAD (NADH) absorbs strongly at 340 nm. The oxidized form of flavin coenzymes absorbs strongly at 450 nm. Thus one can follow the rate of reduction of NAD^+ by observing the increase in theabsorbance at 340 nm and the formation of $FADH_2$ by following the decrease in absorbance at 450 nm.

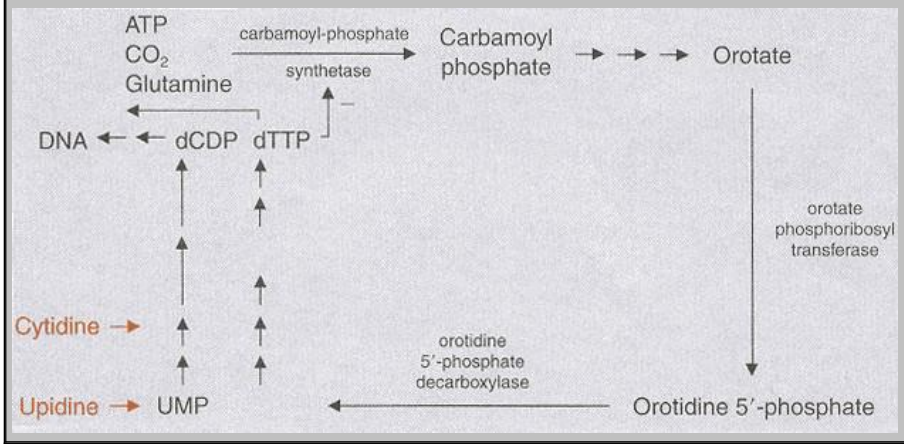
CLINICAL CORRELATION 4.7

Identification and Treatment of an Enzyme Deficiency

Enzyme deficiencies usually lead to increased accumulation of specific intermediary metabolites in plasma and hence in urine. Recognition of the intermediates that accumulate in biological fluids is useful in pinpointing possible enzyme defects. After the enzyme deficiency is established, metabolites that normally occur in the pathway but are distal to the block may be supplied exogenously in order to overcome the metabolic effects of the enzyme deficiency.

In hereditary orotic aciduria there is a double enzyme deficiency in the pyrimidine biosynthetic pathway leading to accumulation of orotic acid. Both orotate phosphoribosyltransferase and orotidine 5 -phosphate decarboxylase are deficient, causing decreased *in vivo* levels of CTP and TTP. The two activities are deficient because they reside in separate domains of a bifunctional polypeptide of 480 amino acids. dCTP and dTTP, which arise from CTP and TTP, are required for cell division. In these enzyme deficiency diseases the patients are pale, weak, and fail to thrive. Administration of the missing pyrimidines as uridine or cytidine promotes growth and general well-being and also decreases orotic acid excretion. The latter occurs because the TTP and CTP formed from the supplied uridine and cytidine repress carbamoyl-phosphate synthetase, the committed step, by feedback inhibition, resulting in a decrease in orotate production.

Webster, D. R., Becroft, D. M. O., and Suttie, D. P. Hereditary orotic aciduria and other diseases of pyrimidine metabolism. In C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed. McGraw-Hill, 1995, p. 1799.



CLINICAL CORRELATION 4.8

Ambiguity in the Assay of Mutated Enzymes

Structural gene mutations leading to production of enzymes with increases or decreases in K_m are frequently observed. A case in point is a patient with hyperuricemia and gout, whose red blood cell hypoxanthine–guanine–phosphoribosyltransferase

(HGPRT) showed little activity in assays *in vitro*. This enzyme is involved in the salvage of purine bases and catalyzes the reaction

$$\text{Hypoxanthine} + \text{PRPP} \rightarrow \text{inosine monophosphate} + PP_i$$

where PRPP is phosphoribosylpyrophosphate.

The absence of HGPRT activity results in a severe neurological disorder known as Lesch–Nyhan syndrome (see p. 499), yet this patient did not have the clinical signs of this disorder. Immunological testing with a specific antibody to the enzyme revealed as much cross-reacting material in the patient's red blood cells as in normal controls. The enzyme was therefore being synthesized but was inactive in the assay *in vitro*. Increasing the substrate concentration in the assay restored full activity in the patient's red cell hemolysate. This anomaly is explained as a mutation in the substrate-binding site of HGPRT, leading to an increased K_m. Neither the substrate concentration in the assay nor in the red blood cells was high enough to bind to the enzyme. This case reinforces the point that an accurate enzyme determination is dependent on zero-order kinetics, that is, the enzyme being saturated with substrate.

Sorenson, L., and Benke, P. J. Biochemical evidence for a distinct type of primary gout. *Nature* 213:1122, 1967.

a specific wavelength of light, A is absorbance, and c is concentration. Absorbance is the log of transmittance (I_0/I). The term ε is a constant that varies from substance to substance; its value can be found in a handbook of biochemistry. In an optically clear solution, the concentration c can be calculated after determination of the absorbance A and substituting into the Beer–Lambert equation.

Many enzymes do not employ NAD^+ or FAD but do generate products that can be utilized by a NAD^+- or FAD-linked enzyme. For example, glucokinase catalyzes the reaction

$$\text{Glucose} + \text{ATP} \leftrightharpoons \text{glucose 6-phosphate} + \text{ADP}$$

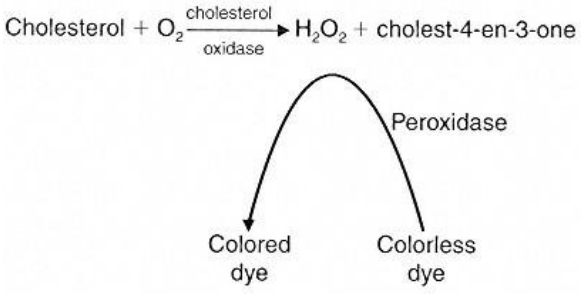
ADP and glucose 6-phosphate (G6P) are difficult to measure directly; however, glucose-6-phosphate dehydrogenase catalyzes the reaction

$$\text{Glucose 6-phosphate} + \text{NADP}^+ \leftrightharpoons \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

Thus by adding an excess of G6P dehydrogenase and $NADP^+$ to the assay mixture, the velocity of production of G6P by glucokinase is proportional to the rate of reduction of $NADP^+$, which can be measured directly in the spectrophotometer.

Clinical Analyzers Use Immobilized Enzymes As Reagents

Enzymes are used as chemical reagents in desk-top clinical analyzers in offices or for screening purposes in shopping centers and malls. For example, screening tests for cholesterol and triacylglycerols can be completed in a few minutes using 10 μL of plasma. The active components in the assay system are cholesterol oxidase for the cholesterol determination and lipase for the triacylglycerols. The enzymes are immobilized in a bilayer along with the necessary buffer salts, cofactors or cosubstrates, and indicator reagents. The ingredients are arranged in a multilayered vehicle the size and thickness of a 35-mm slide. The plasma sample provides the substrate and water necessary to activate the system. In the case of cholesterol oxidase, hydrogen peroxide is a product that subsequently oxidizes a colorless dye to a colored product that is measured by reflectance spectroscopy. Peroxidase is included in the reagents to catalyze the latter reaction.



Each slide packet is constructed to measure a specific substance or enzyme and is stored in the cold for use as needed. In many cases the slide packet contains several enzymes in a coupled assay system that eventually generates a reduced nucleotide or a colored dye that can be measured spectroscopically. This technology has been made possible, in part, by the fact that the enzymes involved are stabilized when bound to immobilized matrices and are stored in the dry state or in the presence of a stabilizing solvent such as glycerol.

Enzyme-Linked Immunoassays Employ Enzymes As Indicators

Modern clinical chemistry has benefited from the marriage of enzyme chemistry and immunology. Antibodies specific to a protein antigen are coupled to an indicator enzyme such as horseradish peroxidase to generate a very specific

and sensitive assay. After binding of the peroxidase-coupled antibody to the antigen, the peroxidase is used to generate a colored product that is measurable and whose concentration is related to the amount of antigen in a sample. Because of the catalytic nature of the enzyme the system greatly amplifies the signal. This assay has been given the acronym **ELISA** for enzyme-linked immunoadsorbent assay.

Application of these principles is demonstrated by an assay for **human immunodeficiency virus (HIV)** coat protein antigens. This virus can lead to development of **acquired immunodeficiency syndrome (AIDS).** Antibodies are prepared in a rabbit against HIV coat proteins. In addition, a reporter antibody is prepared in a goat against rabbit IgG directed against the HIV protein. To this goat anti-rabbit IgG is linked the enzyme, horseradish peroxidase. The test for the virus is performed by incubating patient serum in a polystyrene dish that binds the proteins in the serum sample. Any free protein-binding sites remaining on the dish after incubation with patient serum are then covered by incubating with a nonspecific protein like bovine serum albumin. Next, the rabbit IgG antibody against the HIV protein is incubated in the dish during which time the IgG attaches to any HIV coat proteins that are attached to the polystyrene dish. All unbound rabbit IgG is washed out with buffer. The goat anti-rabbit IgG–peroxidase is now placed in the dish where it binds to any rabbit IgG attached to the dish via the HIV viral coat protein. Unattached antibody–peroxidase is washed out. Peroxidase substrates are added and the amount of color developed in a given time period is a measurement of the amount of HIV coat protein present in a given volume of patient plasma when compared against a standard curve. This procedure is schematically diagrammed in Figure 4.64. This assay amplifies the signal because of the catalytic nature of the reporter group, the enzyme peroxidase. Such amplified enzyme assays allow the measurement of remarkably small amounts of antigens.

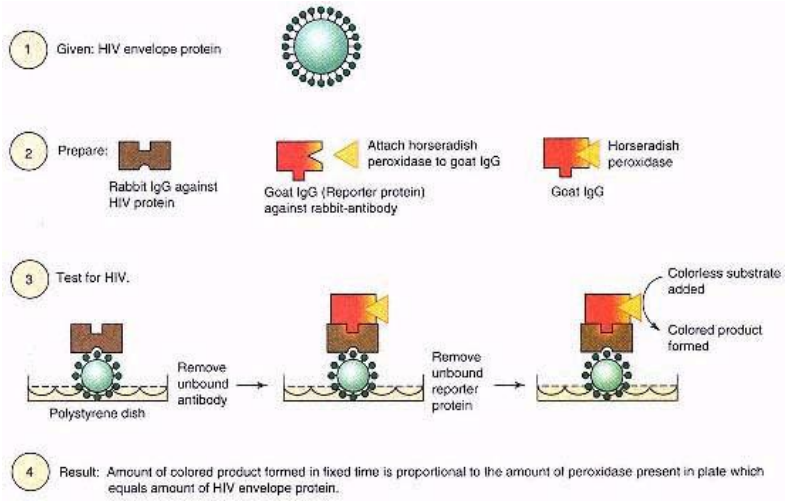


Figure 4.64
Schematic of ELISA (enzyme-linked immunoadsorbent assay) for detecting the human immunodeficiency virus (HIV) envelope proteins.

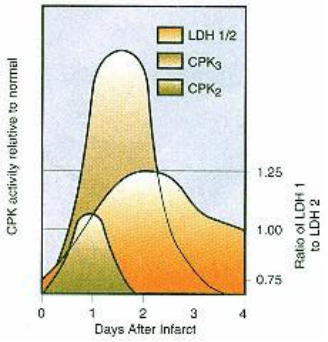


Figure 4.65
Characteristic changes in serum CPK and LDH isozymes following a myocardial infarction.
CPK_2 (MB) isozyme increases to a maximum within 1 day of the infarction. CPK_3 lags behind CPK_2 by about 1 day. Total LDH level increases more slowly. The increase of LDH_1 and LDH_2 within 12–24 h coupled with an increase in CPK_2 is diagnostic of myocardial infarction.

Measurement of Isozymes Is Used Diagnostically

Isozymes (or isoenzymes) are enzymes that catalyze the same reaction but migrate differently on electrophoresis. Their physical properties may also differ, but not necessarily. The most common mechanism for the formation of isozymes involves the arrangement of subunits arising from two different genetic loci in different combinations to form the active polymeric enzyme. Isozymes that have wide clinical application are lactate dehydrogenase, creatine kinase, and alkaline phosphatase. **Creatine kinase (CPK)** (see p. 955) occurs as a dimer with two types of subunits, M (muscle type) and B (brain type). In brain both subunits are electrophoretically the same and are designated B. In skeletal muscle the subunits are both of the M type. The isozyme containing both M and B type subunits (MB) is found only in the myocardium. Other tissues contain variable amounts of the MM and BB isozymes. The isozymes are numbered beginning with the species migrating the fastest to the anode on electrophores—thus, CPK_1 (BB), CPK_2 (MB), and CPK_3 (MM).

Lactate dehydrogenase is a tetrameric enzyme containing only two distinct subunits: those designated H for heart (myocardium) and M for muscle. These two subunits are combined in five different ways. The lactate dehydrogenase isozymes, subunit compositions, and major locations are as follows:

Type	*Composition*	*Location*
LDH_1	HHHH	Myocardium and RBC
LDH_2	HHHM	Myocardium and RBC
LDH_3	HHMM	Brain and kidney
LDH_4	HMMM	
LDH_5	MMMM	Liver and skeletal muscle

To illustrate how kinetic analyses of plasma enzyme activities are useful in medicine, activities of some CPK and LDH isozymes are plotted in Figure 4.65 as a function of time after infarction. After damage to heart tissue the cellular breakup releases CPK_2 into the blood within the first 6–18 h after an infarct, but LDH release lags behind the appearance of CPK_2 by 1 to 2 days. Normally, the activity of the LDH_2 isozyme is higher than that of LDH_1, however, in the case of infarction the activity of LDH_1 becomes greater than LDH_2, at about the time CPK_2 levels are back to baseline (48–60 h). Figure 4.66 shows the fluctuations of all five LDH isozymes after an infarct. The increased ratio of LDH_2 and LDH_1 can be seen in the 24-h tracing. The LDH isozyme "switch" coupled with increased CPK_2 is diagnostic of myocardial infarct (MI) in virtually 100% of the cases. Increased activity of LDH_5 is an indicator of liver congestion. Thus secondary complications of heart failure can be monitored.

The electrophoresis method for determining cardiac enzymes is too slow and insensitive to be of value in the emergency room situation. ELISAs assays based on monoclonal antibodies to CPK_2 are both quick (30 min) and sensitive enough to detect CPK_2 in the serum within an hour or so of a heart attack.

Some Enzymes Are Used As Therapeutic Agents

In a few cases enzymes have been used as drugs in the therapy of specific medical problems. **Streptokinase,** an enzyme mixture prepared from a streptococcus, is useful in clearing blood clots that occur in myocardial infarcts and in the lower extremities. It activates the fibrinolytic proenzyme plasminogen that is normally present in plasma. The activated enzyme is plasmin. **Plasmin** is a serine protease that cleaves the insoluble fibrin in blood clots into several soluble components (see p. 975). Another serine protease, human **tissue plasminogen activator, t-PA,** is being commercially produced by bioengineered

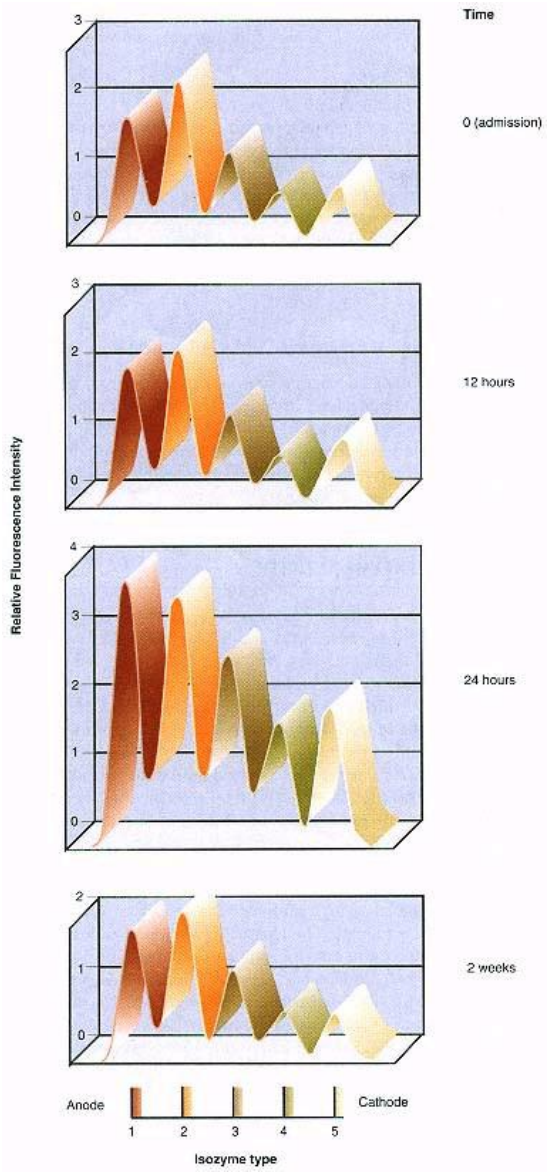


Figure 4.66
Tracings of densitometer scans of LDH isozymes at time intervals following a myocardial infarction.

Total LDH increases and LDH_1 becomes greater than LDH_2 between 12 and 24h. Increase in LDH_5 is diagnostic of a secondary congestive liver involvement. Note the Y axis scales are not identical. After electr ophoresis on agarose gels, the LDH activity is assayed by measuring the fluorescence of the NADH formed in LDH-catalyzed reaction. Courtesy of Dr. A. T. Gajda, Clinical Laboratories, The University of Arkansas for Medical Science.

Escherichia coli (*E. coli*) for use in dissolving blood clots in patients suffering myocardial infarction (see p. 98). t-PA also functions by activating the patient's plasminogen.

Asparaginase therapy is used for some types of adult leukemia. Tumor cells have a requirement for asparagine and must scavenge it from the host's plasma. Intravenous (i.v.) administration of asparaginase lowers the host's plasma level of asparagine, which results in depressing the viability of the tumor.

Most enzymes have a short half-life in blood; consequently, unreasonably large amounts of enzyme are required to maintain therapeutic levels. Work is in progress to enhance enzyme stability by coupling enzymes to solid matrices and implanting these materials in areas that are well perfused. In the future, enzyme replacement in individuals that are genetically deficient in a particular enzyme may be feasible.

Enzymes Linked to Insoluble Matrices Are Used As Chemical Reactors

Specific enzymes linked to insoluble matrices are used in the pharmaceutical industry as highly specific chemical reactors. For example, immobilized β-galactosidase is used to decrease the lactose content of milk for lactose-intolerant people. In production of prednisolone, immobilized steroid 11-β-hydroxylase and a δ-1,2-dehydrogenase convert a cheap precursor to prednisolone in a rapid, stereospecific, and economical manner.

4.10— Regulation of Enzyme Activity

Our discussion up to this point has centered on the chemical and physical characteristics of individual enzymes, but we must be concerned with the physiological integration of many enzymes into a metabolic pathway and the interrelationship of the products of one pathway with the metabolic activity of other pathways. Control of a pathway occurs through modulation of the activity of one or more key enzymes in the pathway. One of the key enzymes is the **rate-limiting** enzyme, which is the enzyme with the lowest V_{max}. It usually occurs early in the pathway. Another is that catalyzing the **committed step** of the pathway, the first irreversible reaction that is unique to a metabolic pathway. The rate-limiting enzyme is not necessarily the enzyme associated with the committed step. Specific examples of these regulatory enzymes will be pointed out in the sections on metabolism.

The activity of the enzyme associated with the committed step or with the rate-limiting step can be regulated in a number of ways. First, the absolute amount of the enzyme can be regulated by change in *de novo* synthesis of the enzyme. Second, the activity of the enzyme can be modulated by activators, by inhibitors, and by covalent modification through mechanisms previously discussed. Finally, the activity of a pathway can be regulated by physically partitioning the pathway from its initial substrate and by controlling access of the substrate to the enzymes of the pathway. This is referred to as **compartmentation.**

Anabolic and catabolic pathways are usually segregated into different organelles in order to maximize the cellular economy. There would be no point to oxidation of fatty acids occurring at the same time and in the same compartment as biosynthesis of fatty acids. If such occurred, a futile cycle would exist. By maintaining fatty acid biosynthesis in the cytoplasm and oxidation in the mitochondria, control can be exerted by regulating transport of common intermediates across the mitochondrial membrane. Table 1.6 (p. 15) contains a compilation of some of the metabolic pathways and their intracellular distribution.

As indicated earlier, the velocity of any reaction is dependent on the amount of enzyme present. Many rate-controlling enzymes are present in very low concentrations. More enzyme may be synthesized or existing rates of synthesis repressed through hormonally instituted activation of the mechanisms controlling gene expression. In some instances substrate can repress the synthesis of enzyme. For example, glucose represses the *de novo* synthesis of pyruvate carboxykinase, which is the rate-limiting enzyme in the conversion of pyruvate to glucose. If there is plenty of glucose available there is no point in synthesizing glucose. This effect of glucose may be mediated via insulin and is not direct feedback inhibition.

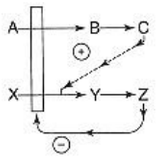


Figure 4.67
Model of feedback inhibition and cross-regulation. Open bar indicates inhibition and broken line indicates activation. Product Z cross-regulates production of C by its inhibitory effect on the enzyme responsible for the conversion of A to B in the A B pathway. C in turn cross-regulates the production of Z. The product Z inhibits its own formation by feedback inhibition of the conversion of X to Y.

Many rate-controlling enzymes have relatively short half-lives; for example, that of pyruvate carboxykinase is 5 h. Teleologically this is reasonable because it provides a mechanism for effecting much larger fluctuations in the activity of a pathway than would be possible by inhibition or activation of existing levels of enzyme.

Short-term regulation occurs through modification of the activity of existing enzyme. For example, when the cellular concentration of deoxyribonucleotides builds up such that the cell has sufficient amounts for synthesis of DNA, the key enzyme of the synthetic pathway is inhibited by the end products, resulting in shutdown of the pathway. This is referred to as **feedback inhibition**. The inhibition may take the form of competitive inhibition or allosteric inhibition. In any case, the apparent K_m may be raised above the *in vivo* levels of substrate, and the reaction ceases or decreases in velocity.

In addition to feedback within the pathway, feedback on other pathways also occurs. This is referred to as **cross-regulation**. In cross-regulation a product of one pathway serves as an inhibitor or activator of an enzyme occurring early in another pathway as depicted in Figure 4.67. A good example, considered in detail in Chapter 12, is the cross-regulation of the production of the four deoxyribonucleotides for DNA synthesis.

An example of reversible covalent modification is glycogen phosphorylase, in which the interconvertible active and inactive forms are phosphorylated and dephosphorylated proteins, respectively. Protein kinases and protein phosphatases are also regulated by phosphorylation and dephosphorylation. Other examples of reversible covalent modification include acetylation–deacetylation, adenylylation–deadenylylation, uridylylation–deuridylylation, and methylation–demethylation. The phosphorylation-dephosphorylation scheme is most common.

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Questions

J. Baggott and C. N. Angstadt

1. In all enzymes the active site:

A. contains the substrate-binding site.

B. is contiguous with the substrate-binding site in the primary sequence.

C. lies in a region of the primary sequence distant from the substrate-binding site.

D. contains a metal ion as a prosthetic group.

E. contains the amino acid side chains involved in catalyzing the reaction.

2. Which of the following types of oxidoreductase enzymes usually form hydrogen peroxide (H_2O_2) as one of their products?

A. dehydrogenases

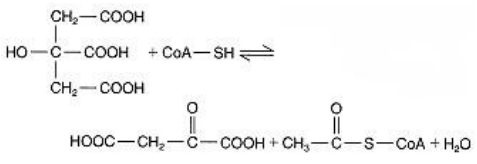
B. oxidases

C. oxygenases

D. peroxidases

E. none of the above

3. The following reaction is catalyzed by:



A. an oxidoreductase.

B. a transferase.

C. a hydrolase.

D. a lyase.

E. a ligase.

4. In the enzyme-catalyzed reaction

$$2\,A + B \rightleftharpoons B + \text{products}$$

where B is the enzyme, when the concentration of A is very high the reaction order is:

A. zero order.

B. first order.

C. second order.

D. third order.

E. a fractional order between first and second.

5. Although enzymic catalysis is reversible, a given reaction may appear irreversible:

A. if the products are thermodynamically far more stable than the reactants.

B. under equilibrium conditions.

C. if a product accumulates.

D. at high enzyme concentrations.

E. at high temperatures.

6. K_m of an enzyme is always:

A. one-half of the V_{max}.

B. a dissociation constant.

C. the normal physiological substrate concentration.

D. the substrate concentration that gives half-maximal velocity.

E. numerically identical for all isozymes that catalyze a given reaction.

7. Cofactors containing the adenosyl group include all of the following EXCEPT:

A. ATP.

B. NAD.

C. NADP.

D. FAD.

E. FMN.

8. Which of the following inhibitor types can be expected to change the K_m of an enzyme but not its V_{max}?

A. competitive

B. noncompetitive

C. uncompetitive

D. irreversible

E. V class allosteric

9. Metal cations may do all of the following EXCEPT:

A. donate electron pairs to functional groups found in the primary structure of the enzyme protein.

B. serve as Lewis acids in enzymes.

C. participate in oxidation–reduction processes.

D. stabilize the active conformation of an enzyme.

E. form chelates with the substrate, with the chelate being the true substrate.

10. Drugs that act as enzyme inhibitors:

A. may function as competitive inhibitors.

B. are clinically useful only when directed against an enzyme unique to a cell that is to be killed.

C. unlike antibiotics, are free of the danger of drug resistance.

D. must be harmless to the patient.

E. generally mimic the three-dimensional structure of the enzyme's active site.

11. Enzymes may be specific with respect to all of the following EXCEPT:

A. chemical identity of the substrate.

B. the atomic mass of the elements in the reactive group (e.g., ^{12}C but not ^{14}C).

C. optical activity of product formed from a symmetrical substrate.

D. type of reaction catalyzed.

E. which of a pair of optical isomers will react.

12. All of the following can be chemically isolated EXCEPT:

A. enzymes.

B. enzyme–substrate complexes.

C. enzyme–inhibitor complexes.

D. enzyme–substrate covalent intermediates.

E. transition states.

13. Which of the following necessarily results in formation of an enzyme–substrate intermediate?

A. substrate strain

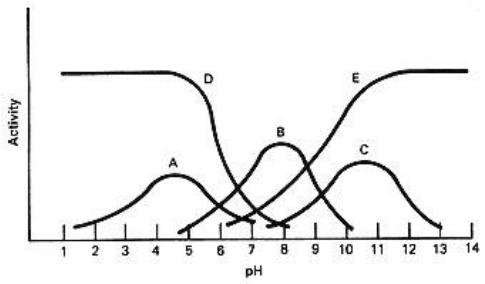
B. acid–base catalysis

C. entropy effects

D. allosteric regulation

E. covalent catalysis

14. An enzyme with histidyl residues that participate in both general acid and general base catalysis would be most likely to have a pH–activity profile resembling which curve on the following drawing?



A. Curve A

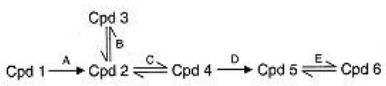
B. Curve B

C. Curve C

D. Curve D

E. Curve E

15. In the reaction sequence below the best point for controlling production of Compound 6 is reaction:



A. A.

B. B.

C. C.

D. D.

E. E.

16. If the plasma activity of an intracellular enzyme is abnormally high, all of the following may be a valid explanation EXCEPT:

A. the rate of removal of the enzyme from plasma may be depressed.

B. tissue damage may have occurred.

C. the enzyme may have been activated.

D. determination of the isozyme distribution may yield useful information.

E. the rate of synthesis of the enzyme may have increased.

17. Types of physiological regulation of enzyme activity include all of the following EXCEPT:

A. covalent modification.

B. changes in rate of synthesis of the enzyme.

C. allosteric activation.

D. suicide inhibition.

E. competitive inhibition.

Answers

1. E The active site contains all the machinery, including the amino acid side chains, involved in catalyzing the reaction. A–D are all possible, but none is necessarily true (p. 129).

2. B Most oxidases produce H_2O_2; as a product of the transfer of two electrons from the donor to oxygen. Typical oxidases are flavoenzymes; the cytochrome oxidase complex is a striking exception (p. 129).

3. D This is an unusually complicated lyase reaction, since secondary reactions are involved. It is a lyase because it removes a group (the acetyl group) with formation of a double bond (the C=O bond of the C4 product, oxalacetate). The common name of this enzyme is citrate synthase (p. 132).

4. A At very high concentration of substrate the enzyme is saturated, and the rate of the reaction is independent of reactant (A) concentration. One could argue that the reaction rate depends on enzyme concentration, but in this situation, where enzyme is regenerated, the system would follow a zero-order rate law (p. 136).

5. A Stable products do not react in the reverse direction at an appreciable rate (p. 135). At equilibrium the forward and reverse reactions proceed at identical rates. Product accumulation would tend to reverse the reaction. Enzymes merely catalyze reactions and do not affect the equilibrium of the reaction (p. 128). Temperature affects the rates of reactions, and may also affect the position of the equilibrium, but does not interconvert reversible and irreversible reactions (p. 135).

6. D This is the experimental definition of K_m. The value can be interpreted as a dissociation constant under certain conditions (p. 139), and often it makes sense that K_m be within the physiological range of substrate concentrations (p. 138).

7. E The A in ATP, NAD, NADP, and FAD refers to an adenylate moiety. It is curious that all these different cofactors incorporate the same group, a group that plays no role in the catalytic process. Presumably its role is in cofactor binding (p. 143).

8. A In the presence of a competitive inhibitor the same V_{max} can be reached, but only if the substrate concentration is increased sufficiently. Effectors of K class allosteric enzymes act like competitive inhibitors, changing K_m but not V_{max}; the opposite is true of V class enzymes (pp. 145 and 152).

9. A Metal cations are electron deficient and may accept electron pairs, serving as Lewis acids, but they do not donate electrons to other functional groups. On the contrary, they sometimes accept electron pairs from groups in amino acid side chains. In doing so they may become chelated (p. 143). Sometimes they are chelated by the substrate, with the chelate being the true substrate (p. 144).

10. A Drugs may serve as competitive inhibitors, such as sulfanilamide (p. 149), or as irreversible inhibitors, such as fluorouracil (p. 151). Pathways unique to pathogenic bacteria, viruses, and so on are rare, so drugs are often developed that are merely less harmful to the host than the target cell because of differences in cell permeability, metabolic rate, and so on (p. 149). Drug resistance can arise through gene amplification in the patient; this can occur with methotrexate (p. 150). Methotrexate is also an example of a drug that is toxic to the patient and must be used with care. Enzyme inhibitors do

not mimic the structure of the active site; rather, they often complement it (p. 156), so that they bind in place of the substrate (p. 148).

11. B Enzymes are specific for the substrate and the type of reaction (p. 154). The asymmetry of the binding site generally permits only one of a pair of optical isomers to react, and only one optical isomer is generated when a symmetric substrate yields an asymmetric product (p. 155). Enzymes do not distinguish among different nuclides of an element, although the rate of reaction of a heavier nuclide might be less than that of a lighter one.

12. E The transition state is not an intermediate and cannot be isolated (p. 159). Rather, it can be thought of as a state in which old bonds are partly broken and new bonds partly formed. All the other species (A–D) can be isolated under suitable experimental conditions.

13. E All enzyme-catalyzed reactions involve an enzyme–substrate complex. There is always at least one transition state involved, but only in covalent catalysis is a covalent bond between enzyme and a portion of the substrate involved (p. 163).

14. B A group must be in the correct ionization state to act catalytically. For a histidyl group to serve as a general acid and a general base (as it does in chymotrypsin), the pH must be compatible with both ionization states of histidine. Since the p*K* of the histidyl side chain is about 6.8, the maximum activity is likely to be near that pH. Chymotrypsin's pH optimum is in the 7–9 range (pp. 160 and 166).

15. D Control of reaction A would control production of Cpd 3 and Cpd 6. Reaction B is not on the direct route. Reaction C is freely reversible, so it does not need to be controlled. Reaction D is irreversible; if it were not controlled, Cpd 5 might build up to toxic levels (p. 174).

16. E Intracellular enzymes may appear in abnormal amounts when tissues are damaged. Different tissues have characteristic distributions of isozymes. Since appearance of intracellular enzymes in plasma arises from leakage, typically from damaged or destroyed cells, changes in their rates of synthesis within the cell would not be expected to affect plasma concentration (p. 166).

17 D Covalent modification includes zymogen activation and phospho–dephospho protein conversions (p. 175). Enzyme levels may be controlled (p. 174). Allosteric activation is common. End products of a reaction or reaction sequence may inhibit their own formation by competitive inhibition (p. 140). Suicide inhibitors are sometimes used as drugs (p. 150).