Chapter 15

Enzymes

15.1 CHARACTERISTICS AND NOMENCLATURE

Enzymes are single- or multiple-chain proteins that act as biological catalysts with the ability to promote specific chemical reactions under the mild conditions that prevail in most living organisms. They have three distinctive characteristics:

- *Specificity:* Enzymes show a characteristic specificity for the reaction they promote and the substrates they can use. As a generalisation, anabolic enzymes show a higher specificity than catabolic ones. Bond specificity is characteristic of enzymes such as the peptidases and esterases that hydrolyse specific bond types. The specificity of these enzymes is determined by the presence of specific functional groups adjacent to the bond to be cleaved. Group specificity is characteristic of enzymes that promote a particular reaction on a structurally related group of substrates. As an example, the kinases catalyse the phosphorylation of substrates that have a common structural feature such as a particular amino acid (e.g. the tyrosine kinases) or sugar (e.g. hexokinase). Absolute or near-absolute specificity is characteristic of anabolic enzymes that catalyse one specific reaction. Enzymes may also display stereospecificity and be able to distinguish between optical and geometrical isomers of substrates or, in the case of the NAD^+- or $NADP^+$-requiring dehydrogenases, to distinguish between apparently identical hydrogen atoms located on opposite sides of the nicotinamide ring (see Section 15.4.4).
- *High catalytic rate:* Enzymes enhance the reaction rate by factors as high as 10^{12} relative to the non-enzyme-catalysed reaction. They achieve this high catalytic power by facilitating the formation of an energetically favoured transition state from substrate to product, thereby lowering the activation energy for the reaction. They do not alter the position of equilibrium of a reversible reaction but they do accelerate the establishment of the equilibrium position (see Section 15.3.5).
- *High capacity for regulation:* The activity of enzymes that control the rate of a particular metabolic pathway can be enhanced or reduced in response to changing intracellular and extracellular demands. A range of regulatory mechanisms operate to allow short-, medium- and long-term changes in activity. Examples include feedback inhibition, covalent modification and gene induction and repression (see Section 15.5.2).

Binding sites

Enzymes reversibly bind their substrate(s) at a specific binding site, generally known as the active or catalytic site, created by the specific three-dimensional structure of the protein molecule but consisting of a relatively small number of amino acid residues. The resulting enzyme–substrate complex promotes a chemical reaction, facilitated by specific amino acid residues in the catalytic site, resulting in the formation of the product. Different amino acid residues in the site may be involved in the binding of the substrate(s) in the correct stereo orientation and in promoting the chemical reaction. An enzyme may also contain regulatory or allosteric sites to which other molecules, commonly distinct from the substrate and often key metabolites such as ATP and AMP, can bind, inducing conformational changes in the active site and thereby enhancing or reducing the activity of the catalytic site. These regulatory molecules are termed effectors, and their regulatory site may be on either the same subunit as the catalytic site or a different subunit (see Section 15.3.3).

The binding of a substrate to an enzyme involves a number of weak, reversible forces including:

- ion-pairing (salt bridges) between ionised groups ($-NH_3^+$, $-COO^-$) within the substrate and oppositely charged groups within the catalytic site;
- hydrogen bonding between such groups as -OH, $-NH_2$, -COOH;
- interatomic van der Waals forces;
- hydrophobic interactions;
- cation-π interactions – electrostatic attraction between a cation, such as that of a protonated amino group within the substrate, and the negative electrostatic potential associated with the face of a simple π system typically provided by the aromatic side-chain of phenylalanine, tyrosine or tryptophan found in the catalytic site.

Cofactors

The catalytic properties of an enzyme are often dependent upon the presence of non-peptide molecules called cofactors or coenzymes. These may be either weakly or tightly bound to the enzyme, in the latter case they are referred to as a prosthetic group. Examples of coenzymes include NAD^+, $NADP^+$, FMN and FAD, whilst examples of prosthetic groups include haem and oligosaccharides, and simple metal ions such as Mg^{2+}, Fe^{2+} and Zn^{2+}. An enzyme lacking its cofactor is termed an apoenzyme, and the active enzyme with its cofactor the holoenzyme.

Nomenclature and classification

By international convention, each enzyme is classified into one of six groups on the basis of the type of chemical reaction that it catalyses. Each group is divided into subgroups according to the nature of the chemical group and coenzymes involved in the reaction. In accordance with the Enzyme Commission (EC) rules, each enzyme can be assigned a unique four-figure code and an unambiguous systematic name based upon the reaction catalysed. The six groups are:

- *Group 1:* Oxidoreductases, which transfer hydrogen or oxygen atoms or electrons from one substrate to another. The group includes the dehydrogenases, reductases, oxidases, dioxidases, hydroxylases, peroxidases and catalase;
- *Group 2:* Transferases, which transfer chemical groups between substrates. The group includes the kinases, aminotransferases, acetyltransferases, carbamyltransferases and phosphorylases.
- *Group 3:* Hydrolases, which catalyse the hydrolytic cleavage of bonds. The group includes the peptidases, esterases, phosphatases and sulphatases.
- *Group 4:* Lyases, which catalyse elimination reactions resulting in the formation of double bonds. The group includes adenylyl cyclase (also known adenylate cyclase), enolase and aldolase.
- *Group 5:* Isomerases, which interconvert isomers of various types by intramolecular rearrangements. The group includes phosphoglucomutase and glucose-6-phosphate isomerase;
- *Group 6:* Ligases (also called synthases), which catalyse covalent bond formation with the concomitant breakdown of a nucleoside triphosphate, commonly ATP. The group includes carbamoyl phosphate synthase and DNA ligase.

As an example of the operation of these rules consider the enzyme alcohol dehydrogenase that catalyses the reaction:

$$\text{alcohol} + \text{NAD}^+ \rightleftharpoons \text{aldehyde or ketone} + \text{NADH} + \text{H}^+$$

It has the systematic name alcohol:NAD oxidoreductase and the classification number 1 : 1 : 1 : 1. The first 1 indicates that it is an oxidoreductase, the second 1 that it acts on a CH-OH donor, the third 1 that NAD^+ or $NADP^+$ is the acceptor and the fourth 1 that it is the first enzyme named in the 1:1:1 subgroup. Systematic names tend to be user-unfriendly and for day-to-day purposes recommended trivial names are preferred. When correctly used they give a reasonable indication of the reaction promoted by the enzyme in question but they fail to fully identify all the reactants involved. For example, glyceraldehydes-3-phosphate dehydrogenase fails to identify the involvement of orthophosphate and NAD^+ and phosphorylase kinase fails to convey the information that it is the b form of phosphorylase that is subject to phosphorylation involving ATP.

Isoenzymes

Some enzymes exist in multiple forms called isoenzymes or isoforms that differ in amino acid sequence. An example is lactate dehydrogenase (LD) (EC 1:1:1:27) that exists in five isoforms. LD is a tetramer that can be assembled from two subunits H (for heart) and M (for muscle). The five forms are therefore H4, H3M, H2M2, HM3 and M4, which can be separated by electrophoresis and shown to have different affinities for their substrates, lactate and pyruvate, and for analogues of these two compounds. They also have different maximum catalytic activities and tissue

distributions, and as a consequence are important in diagnostic enzymology (Section 1.7.3).

Multienzyme complexes

Some enzymes that promote consecutive reactions in a metabolic pathway associate to form a multienzyme complex. Examples include fatty-acid synthase (EC 2:3:1:86) (seven catalytic centres), pyruvate dehydrogenase (EC 2:7:1:99) (three catalytic centres) and DNA polymerase (EC 2:7:7:7) (three catalytic centres). Multienzyme complexes have a number of advantages over individual enzymes, including a reduction in the transit time for the diffusion of the product of one enzyme to the catalytic site of the next, a reduction in the possibility of the product of one enzyme being acted upon by another enzyme not involved in the pathway, and the possibility of one enzyme activating an adjacent enzyme (Section 15.4.7).

Units of enzyme activity

Units of enzyme activity are expressed either in the SI units of katals (moles of substrate consumed or product formed per second) or international units (IU) (μmoles of substrate consumed or product formed per minute). Allied to activity units is specific activity that expresses the number of IU per mg protein or katals per kg protein (note: 60 international units per mg protein is equivalent to 1 katal (kg protein)$^{-1}$).

15.2 ANALYTICAL METHODS FOR THE STUDY OF ENZYME REACTIONS

15.2.1 General considerations

Enzyme assays are undertaken for a variety of reasons, but the two most common are:

- to determine the amount (or concentration or activity) of enzyme present in a particular preparation (this is particularly important in diagnostic enzymology, see Section 1.7.3),
- to gain an insight into the kinetic characteristics of the reaction and hence to determine a range of kinetic constants such as K_m, V_{max} and k_{cat}.

Analytical methods for enzyme assays may be classified as either continuous (kinetic) or discontinuous (fixed-time). Continuous methods monitor some property change (e.g. absorbance or fluorescence) in the reaction mixture, whereas discontinuous methods require samples to be withdrawn from the reaction mixture and analysed by some convenient technique. The inherent greater accuracy of continuous methods commends them whenever they are available.

Initial rates

When an enzyme is mixed with an excess of substrate there is an initial short period of time (a few hundred microseconds) during which intermediates leading to the formation of the product gradually build up (Fig. 15.1). This so-called pre-steady state requires special techniques for study and these are discussed in

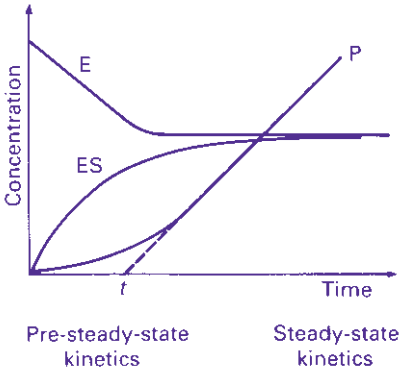


Fig. 15.1. Pre-steady-state progress curve for the interaction of an enzyme (E) with its substrate (S). P, product; *t*, induction time.

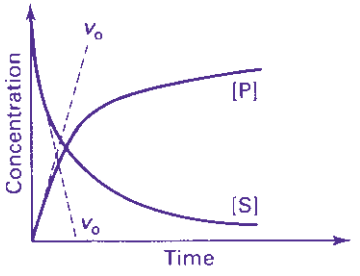


Fig. 15.2. Calculation of initial rate (v_o) from the time-dependent change in the concentration of substrate (S) and product (P) of an enzyme-catalysed reaction.

Section 15.2.3. After this pre-steady state, the reaction rate and the concentration of intermediates change relatively slowly with time and so-called steady-state kinetics exist. Measurement of the progress of the reaction during this phase gives the relationships shown in Fig. 15.2. Tangents drawn through the origin to the curves of substrate concentration and product concentration versus time allow the initial rate, v_o, to be calculated. This is the maximum rate for a given concentration of enzyme and substrate under the defined experimental conditions. Measurement of the initial rate of an enzyme-catalysed reaction is a prerequisite to a complete understanding of the mechanism by which the enzyme works, as well as to the estimation of the activity of an enzyme in a biological sample. Its numerical value is influenced by many factors, including substrate and enzyme concentration, pH, temperature and the presence of activators or inhibitors.

For simplicity, initial rates are sometimes determined experimentally on the basis of a single measurement of the amount of substrate consumed or product produced in a given time rather than by the tangent method. This approach is valid over only the short period of time when the reaction is proceeding effectively at a constant rate. This linear rate section comprises at the most the first 10% of the total possible change and clearly the error is smaller the earlier the rate is measured. In such cases, the initial rate is proportional either to the reciprocal of

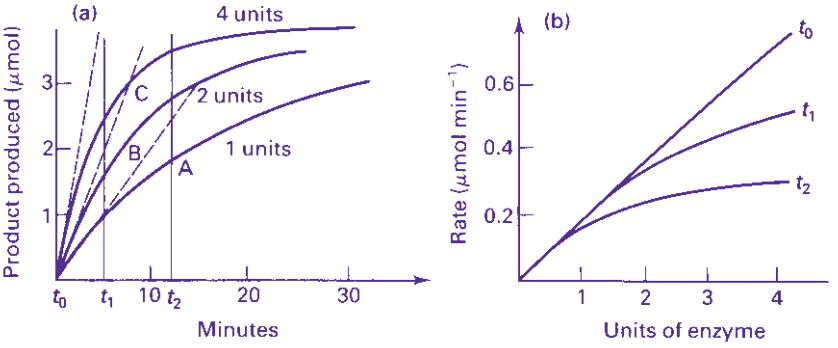


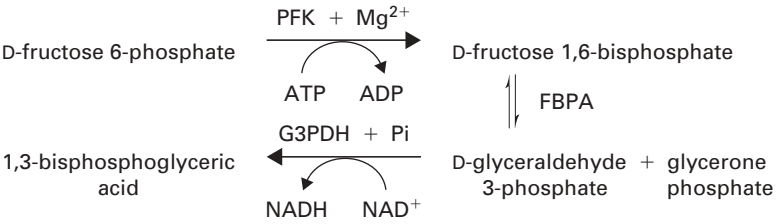
Fig. 15.3. The importance of measuring the initial rate in the assay of an enzyme. (a) Time-dependent variation in the concentration of products in the presence of (A)1, (B)2 and (C)4 units of enzyme; (b) variation of reaction rate with enzyme concentration using true initial rate (v_o) and two fixed time assays (t_1 and t_2).

the time to produce a fixed change (fixed-change assays) or to the amount of substrate reacted in a given time (fixed-time assays). The potential problem with fixed-time assays is illustrated in Fig. 15.3, which represents the effect of enzyme concentration on the progress of the reaction in the presence of a constant initial substrate concentration (Fig. 15.3a). Measurement of the rate of the reaction at time t_0 (by the tangent method) to give the true initial rate or at two fixed times, t_1 and t_2, gives the relationship between initial rate and enzyme concentration shown in Fig. 15.3b. It can be seen that only the tangent method gives the correct linear relationship. Since the correct determination of initial rate means that the observed changes in the concentration of substrate or product are relatively small, it is inherently more accurate to measure the increase in product concentration because the relative increase in its concentration is significantly larger than the corresponding decrease in substrate concentration.

15.2.2 Analytical methods for steady-state studies

Visible and ultraviolet spectrophotometric methods

Many substrates and products absorb light in the visible or ultraviolet region and the change in the absorbance during the reaction can be used as the basis for the enzyme assay. It is essential that the substrate and product do not absorb at the same wavelength and that the Beer–Lambert law (Section 12.4.1) is obeyed for the chosen analyte. A large number of common enzyme assays are based on the interconversion of $NAD(P)^+$ and NAD(P)H. Both of these nucleotides absorb at 260 nm but only the reduced form absorbs at 340 nm. Enzymes that do not involve this interconversion can be assayed by means of a coupled reaction, which involves two enzyme reactions linked by means of common intermediates. The assay of 6-phosphofructokinase (PFK) (EC 2:7:1:11) coupled to fructose-bisphosphatase aldolase (FBPA) (EC 4:1:2:13) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (EC 1:2:1:12) illustrates the principle:



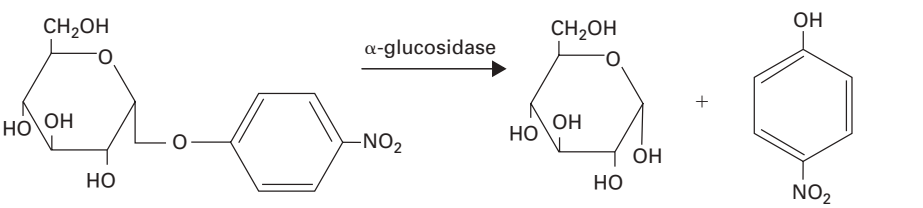
The assay mixture would contain D-fructose-6-phosphate, ATP, Mg^{2+}, FBPA, G3PDH, NAD^+ and P_i all in excess so that the reaction would go to completion and the rate of reduction of NAD^+ and the production of NADH, and hence the increase in absorbance at 340 nm, would be determined solely by the activity of PFK added to the reaction mixture in a known volume of the test enzyme preparation. In principle there is no limit to the number of reactions that can be coupled in this way provided that the enzyme under investigation is always present in limiting amounts.

The number of units of enzyme in the test preparation can be calculated by applying the Beer–Lambert law to calculate the amount of product formed per second:

$$\text{enzyme units (katal per cm}^3\text{ test solution)} = \frac{\Delta E_{340}}{\varepsilon_\lambda} \times \frac{a}{1000} \times \frac{1000}{x}$$

where ΔE_{340} is the control-corrected change in the absorbance at 340 nm per second, a is the total volume (cm^3) of reaction mixture (generally about 3 cm^3) in a cuvette of 1 cm light path, x is the volume (mm^3) of test solution added to the reaction mixture, and ε_λ is the molar extinction coefficient for NADH at 340 nm (6.3×10^3 $(mol\ dm^{-3})^{-1}\ cm^{-1}$). By dividing the above equation by the total concentration of protein in the test enzyme preparation, the specific activity (katal per kg) of the preparation can be calculated.

The scope of visible spectrophotometric enzyme assays can be extended by the use of synthetic substrates that release a coloured product. Many such artificial substrates are available commercially, particularly for the assay of hydrolytic enzymes. The favoured coloured products are phenolphthalein and *p*-nitrophenol, both of which are coloured in alkaline solution. An example is the assay of α-D-glucosidase (maltase) (EC 3:2:1:20):



p-nitrophenyl-α-D-glucopyranoside D-glucose *p*-nitrophenol (yellow)

An extension of this approach is the use of synthetic dyes for the study of oxidoreductases. The oxidised and reduced forms of these dyes have different colours. Examples are the tetrazolium dyes, methylene blue, 2,6-dichlorophenol

indophenol, and methyl and benzyl viologen (Table 1.8). Their use is dependent upon them having an appropriate redox potential (Section 1.5.1), relative to that of the substrate, so that the free energy change for the reaction is negative, allowing the reaction to proceed in the required direction.

Spectrofluorimetric methods

Fluorimetric enzyme assays have the significant practical advantage that they are highly sensitive and can therefore detect and measure enzymes at low concentrations. However, they are sensitive to traces of impurities in the enzyme preparation that can quench the emitted radiation. Additionally, some fluorescent compounds are unstable, especially in the presence of ultraviolet light. NAD(P)H is fluorescent and so enzymes utilising it can be assayed either by their absorption at 340 nm or by their fluorescence (primary wavelength 340 nm, reference wavelength 378 nm). Synthetic substrates that release a fluorescent product are also available for the assay of some enzymes. An example is the assay of β-D-glucuronidase (EC 3:2:1:31) using 4-methlyumbelliferyl-β-D-glucuronide as the substrate that releases the fluorescent product 4-methylumbelliferone.

Luminescence methods

Bioluminescence reactions are commonly used as the basis for an enzyme assay owing to their high sensitivity. The assay of luciferase is an example:

$$\text{luciferin} + \text{ATP} + \text{O}_2 \xrightleftharpoons{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PP}_i + \text{CO}_2 + \textit{light}$$

The assay can be used to assay ATP and enzymes that utilise ATP by means of coupled reactions. An example is the assay of malate dehydrogenase (MD) (EC 1:1:1:37). This coupled assay is based on the fact that bacterial luciferase uses reduced FMN to oxidise long-chain aliphatic aldehydes (RCHO):

$$(S)\text{-malate} + \text{NAD}^+ \xrightleftharpoons{\text{MD}} \text{oxaloacetate} + \text{NADH} + \text{H}^+$$

$$\text{NADH} + \text{H}^+ + \text{FMN} \xrightleftharpoons{\text{oxidoreductase}} \text{FMNH}_2 + \text{NAD}^+$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightleftharpoons{\text{luciferase}} \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \textit{light}$$

The use of excess reagents would ensure that each reaction went to completion.

Immunochemical methods

Monoclonal antibodies raised to a particular enzyme can be used as a basis for a highly specific enzyme-linked immunosorbent assay (ELISA) for the enzyme. Such assays can distinguish between isoenzyme forms, which make them attractive for diagnostic purposes. An important clinical example is creatine kinase (CK). It is a dimer based on two different subunits, M and B. The MB isoenzyme is important in the diagnosis of myocardial infarction (heart attack) and an immunological assay is important in its assay. Further details of the assay of CK-MB are given in Section 1.7.3. The principles of this type of assay are discussed in Section 7.7.

Ion-selective and oxygen electrode methods

The availability of ion-selective electrodes (ISEs) and gas-sensing electrodes, such as those for the ammonium ion, oxygen, carbon dioxide and ammonia have allowed attractive methods to be developed for many enzyme assays in which these species are consumed or released. The methods are sensitive, reproducible and can be applied to very small volumes of test solution (Section 1.5.2).

Radioisotope methods

Although potentially very sensitive, radioisotope methods for enzyme assays are restricted to applications where it is possible to separate easily the radiolabelled forms of the substrate and product. It is most commonly applied to cases in which one of the products is a gas. The assay of glutamate decarboxylase (GD) is an example:

$$\underset{\text{L-glutamic acid}}{HOOCCH_2CH(NH_2)^{14}COOH} \xrightleftharpoons{GD} {}^{14}CO_2 + \underset{\gamma\text{-aminobutyric acid}}{HOOCCH_2CH_2CH_2NH_2}$$

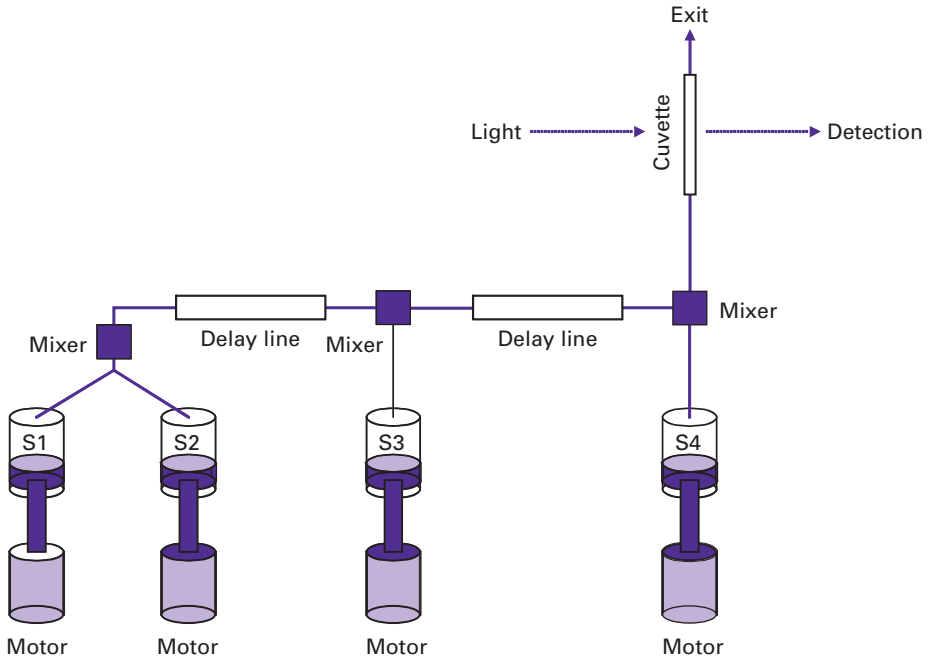
The $^{14}CO_2$ evolved is trapped in alkali and hence the rate of $^{14}CO_2$ evolution measured. In other cases not involving the evolution of a gas, the substrate and product can be separated by a solvent extraction technique but this does not lend itself to routine analysis. GD can also be assayed using a CO_2 electrode.

Practical considerations

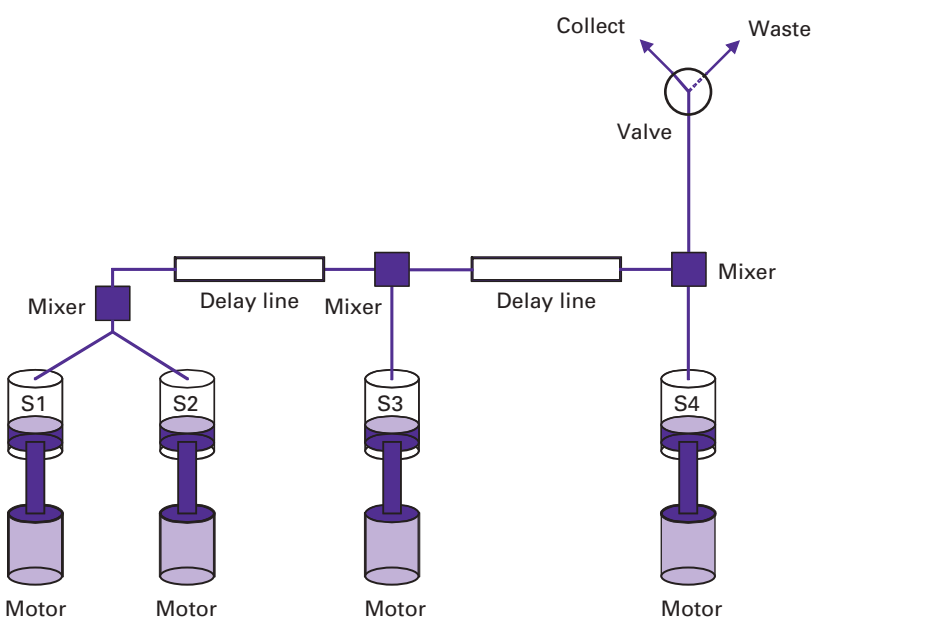
Once the analytical method for a particular assay has been selected, a number of practical issues should be considered:

- All reactants should be of a high purity and all apparatus should be scrupulously clean.
- Variables such as pH, temperature and ionic strength should be controlled.
- All studies should include an appropriate control that is in all respects the same as the test assay but lacking either enzyme or substrate. Changes in the experimental parameter in the control lacking the test enzyme will give an assessment of the extent of the non-enzymatic reaction whereas changes in the control lacking added substrate will evaluate any background reaction in the enzyme preparation. It is worthwhile assaying the enzyme using different volumes of the test solution to confirm linearity between initial rate and enzyme concentration, thereby confirming the absence of activators or inhibitors in the preparation.
- Enzyme assays should be carried out with excess substrate (at least 10 K_m (see Section 15.3)).
- Kinetic studies should be carried out with substrate concentrations ranging from 0.1 to 10 K_m.
- Most assays are carried out at 30 °C but some are performed at 37 °C because of the physiological significance of the temperature.

SFM-400 Stopped-flow mode



SFM-400 Quenched-flow mode



- the assay method should be appropriately calibrated and validated using the principles discussed in Section 1.6.5.

15.2.3 Analytical methods for pre-steady-state studies

The experimental techniques discussed in the previous section are not suitable for the study of the progress of enzyme reactions in the short period of time (commonly milliseconds) before steady-state conditions, with respect to the formation of enzyme–substrate complex, are established. Fig. 15.1 shows the progress curves for this pre-steady-state initial stage of an enzymatic reaction. The induction time t is related to the rate constants for the formation and dissociation of the enzyme–substrate (ES) complex. Two main types of method are available for the study of this pre-steady state.

Rapid mixing methods

In the continuous flow method, separate solutions of the enzyme and substrate are introduced from syringes, each of 10 cm^3 maximum volume, into a mixing chamber typically of 100 mm^3 capacity. The mixture is then pumped at a pre-selected speed through a narrow tube that is illuminated by a light source and monitored by a photomultiplier detector. Flow through the tube is fast, typically 10 $m\,s^{-1}$, so that it is turbulent, thus ensuring that the solution is homogeneous. The precise flow time from the time of mixing to the observation point can be calculated from the known flow rate. By varying the flow rate the reaction time at the observation point can be varied, allowing the extent of reaction to be studied as a function of time. From these data the various rate constants can be calculated. The technique uses relatively small amounts of reactants and is limited only by the time required to mix the two reactants.

The stopped-flow method is a variant of the continuous-flow method in that shortly after the reactants emerge from the mixing chamber the flow is stopped and the detector triggered to continuously monitor the change in the experimental parameter such as absorbance or fluorescence (Fig. 15.4). Special flow cells are used together with a detector that allows readings to be taken 180° to the light source for absorbance, transmittance or circular dichroism measurements, or at 90° to the source for fluorescence, fluorescence anisotropy or light-scattering measurements.

Fig. 15.4. The Bio-Logic stopped-flow (top) and quenched-flow (bottom) apparatus. The reactants are placed in separate syringes each driven by a microprocessor-controlled stepping motor capable of delivering 0.01 to 10.00 $cm^3\,min^{-1}$ with a minimum injection volume of 10–30 mm^3. The reactants are pre-mixed before they enter the delay line (variable volume between 25 and 1000 mm^3) and then the flow cell cuvette with a minimum dead time of 0.6 ms. The flow can be stopped at any predetermined time either by stopping the stepping motor or by closing the outlet from the reaction cuvette. The reaction can be studied by visible, ultraviolet, fluorescence or circular dichroism spectroscopy. The optical path length can be varied between 0.8 and 10 mm. In quench-flow mode the minimum ageing time is <2 ms. The quenching agent is added from the third or fourth syringe. (Reproduced by permission of BioLogic Science Instruments, France: website <www.bio-logic.info>.)

It is also possible to take measurements by mass spectrometry, X-ray scattering and conductivity. The attraction of the method is its conservation of reactants.

A variant of the stopped flow method is the quenching method. In this technique the reactants from the mixing chamber are treated with a quenching agent from a third syringe. The quenching agent, for example trichloroacetic acid, stops the reaction, which is then monitored by an appropriate analytical method for the build-up of intermediates. By varying the time between mixing the reactants and adding the quenching reagent, the kinetics of this build-up can be studied. A disadvantage of this approach is that it uses more reactants than the stopped-flow method, since the kinetic data are acquired from a series of studies rather than by following one reaction for a period of time. Both methods have difficulty in monitoring the first millisecond of reaction owing to the need to allow mixing to take place, but this problem can be partly solved by changing the pH or temperature in order to slow down the reaction. Both methods commonly use synthetic substrates that release a coloured product or give rise to a coloured acyl or phosphoryl intermediate.

Relaxation methods

The limitation of the stopped-flow method is the dead time during which the enzyme and substrate are mixed. In the relaxation methods an equilibrium mixture of the reactants is preformed and the position of equilibrium altered by a change in reaction conditions. The most common procedure for achieving this is the temperature jump technique in which the reaction temperature is raised rapidly by 5–10 deg.C by the discharge of a capacitor or infrared laser. The rate at which the reaction mixture adjusts to its new equilibrium (relaxation time τ, generally a few microseconds) is inversely related to the rate constants involved in the reaction. This return to equilibrium is monitored by one or more suitable spectrophotometric methods. The recorded data enables the number of intermediates to be deduced and the various rate constants calculated from the relaxation times.

These pre-steady-state techniques have shown that the enzyme and its substrate(s) associate very rapidly, with second-order rate constants for the formation of ES in the range 10^6–10^8 $(\text{mol dm}^{-3})^{-1}\text{s}^{-1}$ and first-order rate constants for the dissociation of ES in the range 10–10^4 s^{-1}. The association process is slower than that predicted by simple collision theory and confirms the need for the specific orientation of the substrate and enzyme, with subsequent conformational changes in the protein and probably the involvement of solvation processes.

The stopped-flow and quenching methods have also been used to study other biochemical processes that are kinetically fast and may involve transient intermediates. For example, the stopped-flow method has been applied to the study of protein folding, protein conformational changes and receptor–ligand binding, and the quenching method to the study of second-messenger studies.

15.2.4 Analytical methods for *in vivo* studies

In the extrapolation of *in vitro* kinetic data to the *in vivo* situation the assumption is made that intracellular compartments such as the cytosol and the mitochondrial

matrix can be regarded as homogeneous bags of enzymes in which the individual enzyme, their substrates and products can diffuse freely. Support for this view originates from cell fractionation studies in which the great proportion of total cell proteins is released when cells are disrupted. However, there is now good evidence, obtained from studies with high voltage electron microscopy (HVEM), that mammalian cells contain an extensive network of interconnecting strands, termed the microtrabecular lattice (MTL), which appears to connect all the intracellular structures. Critics of the existence of the MTL have argued that such structures are artefacts, but there is increasing acceptance that MTL or closely related structures do represent a good approximation of the structure *in vivo*. The consequence of such a model is that previously regarded freely diffusing enzymes are more likely to be loosely bound to the MTL. Such a view gives rise to the concept of the wide existence of multienzyme complexes previously regarded as an exception to the norm. If these views are correct, then the concept arises of the channelling of products from one enzyme to the next (Section 15.4.7). Channelling could occur by a number of mechanisms including: the sequential covalent binding of intermediates to active sites; site-to-site transfer of non-covalently bound intermediates (so-called tight channelling), the transfer of intermediates in an unstirred aqueous layer and the prevention of diffusion of intermediates by electrostatic forces. It has been argued that channelling of metabolic intermediates within an organised enzyme complex could lead to increased flux (rate of utilisation of substrates) through the pathway and restriction of flux in competing pathways. Whether or not the loose association of enzymes will result in a metabolically significant change in the kinetic properties of these enzymes relative to the properties in free solution is still a matter of research.

The most successful analytical technique for studying enzymology in individual cells and in whole organisms is nuclear magnetic resonance spectroscopy (NMR). This non-invasive technique allows the measurement of steady-state metabolite concentrations and of metabolic flux using simple proton NMR, or the redistribution of a ^{13}C label among glycolytic intermediates or the use of ^{31}P NMR to measure ATP turnover and flux. Evidence for enzyme–enzyme interaction has been obtained by studying conformational changes in the enzyme protein. This approach requires the protein to be labelled in some appropriate way. One of the most attractive methods is to insert ^{19}F into the molecule. From an NMR point of view this is an excellent label, since it is an isotope that has a spin quantum number of $\frac{1}{2}$ and therefore has two spin states that are readily studied by NMR. The chemical shift change of the fluorine nucleus is large, making it very sensitive to its local environment in the protein. Moreover, its size is very similar to that of a proton, so that it is unlikely to modify the enzyme's structure. Since fluorine is very rare in biological systems, the NMR signal from the label can be interpreted unambiguously. By studying the relaxation times associated with the fluorine nucleus it is possible to detect restricted motion of the enzyme in a cell due to protein–protein aggregation.

A complementary approach to these NMR studies is that of genetic manipulation. Using molecular biology techniques, it is possible to delete, raise or lower the intracellular concentration of selected enzymes and to study the effect on enzyme

kinetics and metabolic flux. The approach simply requires the availability of cDNA or a genomic clone for the selected enzyme, coupled with gene disruption and anti-sense RNA methodologies.

15.2.5 Analytical methods for substrate assays

Enzyme-based assays are very convenient methods for the estimation of the amount of substrate present in a biological sample. The principle of using excess enzyme (i.e. the substrate concentration should be less than the K_m (see Section 15.3)) and relating the substrate concentration in the test solution to the observed initial rate can be used. It is essential that the reaction goes to completion in a relatively short time. If the reaction is freely reversible, then it is necessary to change the experimental conditions, such as pH or by chemically trapping the product, so that the reaction does approach completion. Coupled reactions are commonly used in substrate assays and they have the attraction that they help in the displacement of reversible reactions. The sensitivity of this initial rate method to substrate assay depends upon the value of the molar extinction coefficient for the analyte being assayed and also on the K_m for the substrate. In practice these two factors place a constraint on the level of substrate that can be assayed. Several approaches are available to overcome this problem. The end-point technique avoids the measurement of initial rate by converting all the substrate to product and then computing the amount present by correlating it with the total change in parameter such as absorbance or fluorescence. The sensitivity of an assay can also be significantly increased by the technique of enzymatic cycling. In this method the substrate is regenerated by means of a coupled reaction and the total change in absorbance, etc., in a given time measured. Precalibration, using a range of substrate concentrations with all the other reactants in excess, allows the substrate concentration in a test solution to be computed. This method has a 10^4- to 10^5-fold increase in sensitivity relative to the end-point technique.

Enzyme-based assays are commonly used in clinical biochemistry to measure substrates in biological samples. For example, the three most common assays for serum glucose are those based on the use of hexokinase, glucose oxidase and glucose dehydrogenase. The first two are based on the coupled reaction technique:

- *Hexokinase method:* This couples the reaction to that of glucose-6-phosphate dehydrogenase and measures the absorbance at 340 nm due to NADH:

$$\text{D-glucose} + \text{ATP} \rightleftharpoons \text{D-glucose 6-phosphate} + \text{ADP}$$
$$\text{D-glucose 6-phosphate} + \text{NAD}^+ \rightleftharpoons \text{D-glucono-1,5-lactone 6-phosphate} + \text{NADH} + \text{H}^+$$

- *Glucose oxidase method:* This couples the reaction to peroxidase and measures the absorption of the oxidised dye in the visible region or uses an oxygen electrode to measure the oxygen consumption directly:

$$\text{D-glucose} + \text{H}_2\text{O} + \text{O}_2 \rightleftharpoons \text{D-gluconic acid} + \text{H}_2\text{O}_2$$
$$\text{H}_2\text{O}_2 + \text{dye}_{\text{reduced}} \rightleftharpoons \text{H}_2\text{O} + \text{dye}_{\text{oxidised}}$$

The glucose oxidase method uses β-D-glucose as substrate but blood glucose contains an equilibrium mixture of it and the α-isomer. Fortunately, preparations of glucose oxidase contain an isomerase that interconverts the two isomers thus allowing the assay of total D-glucose.

- *Glucose dehydrogenase method:* This requires no coupled reaction, but simply measures the increase in absorption at 340 nm:

$$\beta\text{-D-glucose} + NAD^+ \rightleftharpoons \text{D-glucono-1,5-lactone} + NADH + H^+$$

Other blood or urine substrates commonly assayed by enzyme-based techniques commonly used in clinical biochemistry include the following:

- *Creatinine:* This is used as an indicator of kidney function, and uses creatininase, creatine kinase, pyruvate kinase and lactate dehydrogenase:

$$\begin{aligned} \text{creatinine} + H_2O &\rightleftharpoons \text{creatine} \\ \text{creatine} + ATP &\rightleftharpoons \text{phosphocreatine} + ADP \\ ADP + \text{phosphoenolpyruvate} &\rightleftharpoons ATP + \text{pyruvate} \\ \text{pyruvate} + NADH + H^+ &\rightleftharpoons \text{L-lactate} + NAD^+ \end{aligned}$$

- *Cholesterol:* This is used as an indicator of atherosclerosis and susceptibility to coronary heart disease, uses cholesterol oxidase and peroxidase and measures the absorption in the visible region due to the oxidised dye:

$$\begin{aligned} \text{cholesterol} + O_2 &\rightleftharpoons \text{4-cholesten-3-one} + H_2O_2 \\ H_2O_2 + \text{dye}_{\text{reduced}} &\rightleftharpoons H_2O + \text{dye}_{\text{oxidised}} \end{aligned}$$

Any cholesterol ester in the sample is hydrolysed to free cholesterol by the inclusion of cholesterol esterase in the reaction mixture.

Biosensors, such as those for glucose and cholesterol (Section 1.5.2) are based on the above reactions and afford a simple means for the fast measurement of these substrates.

15.3 ENZYME STEADY-STATE KINETICS

15.3.1 Monosubstrate enzyme reactions

For many enzymes, the initial rate, v_o, varies hyperbolically with substrate concentration for a fixed concentration of enzyme (Fig. 15.5). The mathematical equation expressing this hyperbolic relationship between initial rate and substrate concentration is known as the Michaelis–Menten equation:

$$v_o = \frac{V_{max}[S]}{K_m + [S]} \tag{15.1}$$

where V_{max} is the limiting value of the initial rate when all the active sites are occupied, K_m is the Michaelis constant, and [S] is the substrate concentration. At low substrate concentrations the occupancy of the active sites on the enzyme molecules is low and the reaction rate is directly related to the number of sites

Example 1 ENZYMATIC ASSAY OF GLUCOSE

Question

The concentration of glucose in a test solution was assayed using hexokinase and glucose-6-phosphate dehydrogenase. These enzymes in the presence of NAD^+ and Mg^{2+} convert glucose to glucono-1,5-lactone-6-phosphate. If an excess of NAD^+ and enzymes is used, the equilibrium reaction is displaced to the right and essentially goes to completion. A 1.0 cm^3 portion of the test solution was taken and to it was added 1.0 cm^3 of a solution containing excess NAD^+, the two enzymes and $MgCl_2$. The change in absorption at 340 nm was measured in a 1 cm cuvette over a period of time. A maximum absorption change of 0.61 was observed. What was the original concentration of glucose in the test solution given that the molar extinction coefficient of NADH is 6.22×10^3 $(mol\ dm^{-3})^{-1}\ cm^{-1}$ at 340 nm?

Answer

By using excess NAD^+ and the two linked enzymes, all the glucose in the test solution would be consumed and the absorption at 340 nm would be a measure of the amount of NADH produced. This is the only reactant or product that absorbs at 340 nm and as it is stoichiometrically related to the consumption of glucose, its concentration can be calculated.

Applying the Beer–Lambert law (Section 12.4.1, equation 12.5):

$$A = \varepsilon_\lambda c l$$

$$0.61 = 6.22 \times 10^3 \times c \times 1$$

Therefore

$$c = \frac{0.61}{6.22 \times 10^3 \times 1} = 9.807 \times 10^{-5}\ \mathrm{M}$$

However, the original test solution was diluted 1:1 when the reagents were mixed so that the original concentration must have been twice this calculated value, making the original test solution of glucose 1.96×10^{-4} M.

occupied. This approximates to first-order kinetics in that the rate is proportional to substrate concentration. At high substrate concentrations effectively all of the active sites are occupied and the reaction becomes independent of the substrate concentration, since no more enzyme–substrate complex, ES, can be formed and zero-order or saturation kinetics are observed. Under these conditions the reaction rate is dependent upon the conversion of the enzyme substrate complex to products and the diffusion of the products from the enzyme.

It can be seen from equation 15.1, that when $v_o = 0.5 V_{max}$, $K_m = [S]$. Thus K_m is numerically equal to the substrate concentration at which the initial rate is one-half of the maximum rate (Fig. 15.5) and has units of molarity. Values of K_m are usually in the range 10^{-2} to 10^{-5} M and are important because they enable the concentration of substrate required to saturate all of the active sites of the enzyme in an enzyme assay to be calculated. When $[S] \gg K_m$, equation 15.1 reduces to $v_o \approx V_{max}$, but a simple calculation reveals that when $[S] = 10 V_{max}$, v_o is only 90% V_{max} and that when $[S] = 100 K_m$, $v_o = 99\% V_{max}$. Appreciation of this relationship is vital in enzyme assays.

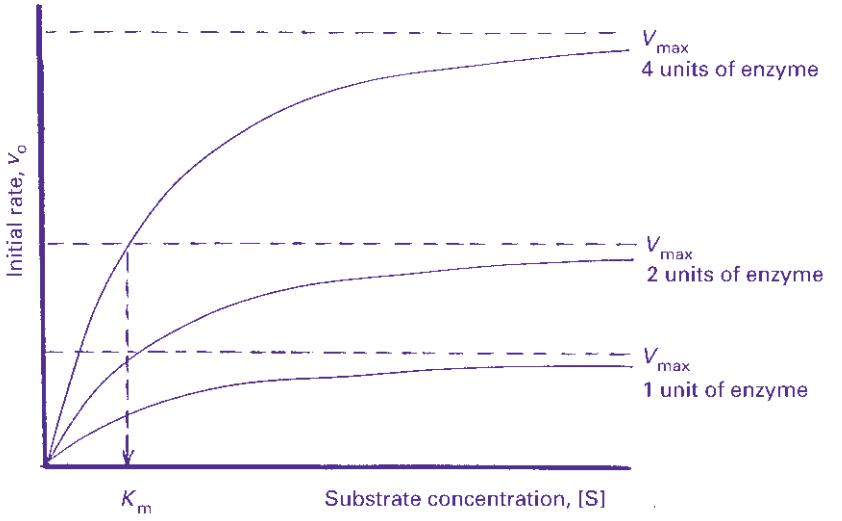


Fig. 15.5. The effect of substrate concentration on the initial rate of an enzyme-catalysed reaction in the presence of three different concentrations of enzyme. Doubling the enzyme concentration doubles the maximum initial rate, V_{max}, but has no effect on K_m.

As previously stated, enzyme-catalysed reactions proceed via the formation of an enzyme–substrate complex in which the substrate (S) is non-covalently bound to the active site of the enzyme (E). The formation of this complex for the majority of enzymes is rapid and reversible and is characterised by the dissociation constant, K_s, of the complex:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$$

where k_{+1} and k_{-1} are the rate constants for the forward and reverse reactions. At equilibrium, the rates of the forward and reverse reactions are equal and the law of mass action can be applied to the reversible process:

$$k_{+1}[E][S] = k_{-1}[ES]$$

hence:

$$K_s = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_{+1}} = \frac{1}{K_a} \tag{15.2}$$

where K_a is the association (or affinity) constant.

It can be seen that when K_s is numerically large, the equilibrium is in favour of unbound E and S, i.e. of non-binding, whilst, when K_s is numerically small, the equilibrium is in favour of the formation of ES, i.e. of binding. Thus K_s is inversely proportional to the affinity of the enzyme for its substrate.

The conversion of ES to product (P) can be most simply represented by the irreversible equation:

$$\mathrm{ES} \xrightarrow{k_{+2}} \mathrm{E} + \mathrm{P}$$

where k_{+2} is the first-order rate constant for the reaction.

In some cases the conversion of ES to E and P may involve several stages and may not necessarily be essentially irreversible. The rate constant k_{+2} is generally smaller than both k_{+1} and k_{-1} and in some cases very much smaller. In general, therefore, the conversion of ES to products is the rate-limiting step such that the concentration of ES is essentially constant but not necessarily the equilibrium concentration. Under these conditions the Michaelis constant, K_m, is given by:

$$K_m = \frac{k_{+2} + k_{-1}}{k_{+1}} = K_s + \frac{k_{+2}}{k_{+1}} \tag{15.3}$$

It is evident that, under these circumstances, K_m must be numerically larger than K_s and only when k_{+2} is very small do K_m and K_s approximately equal each other. The relationship between these two constants is further complicated by the fact that, for some enzyme reactions, two products are formed sequentially, each controlled by different rate constants:

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \xrightarrow{k_{+2}} \mathrm{P_1} + \mathrm{EA} \xrightarrow{k_{+3}} \mathrm{E} + \mathrm{P_2}$$

where P_1 and P_2 are products, and A is a metabolic product of S that is further metabolised to P_2.

In such circumstances it can be shown that:

$$K_m = K_s \frac{(k_{+3})}{(k_{+2} + k_{+3})} \tag{15.4}$$

so that K_m is numerically smaller than K_s. It is obvious therefore that care must be taken in the interpretation of the significance of K_m relative to K_s. Only when the complete reaction mechanism is known can the mathematical relationship between K_m and K_s be fully appreciated and any statement made about the relationship between K_m and the affinity of the enzyme for its substrate.

Although the Michaelis–Menten equation can be used to calculate K_m and V_{max}, its use is subject to error owing to the difficulty of experimentally measuring initial rates at high substrate concentrations and hence of extrapolating the hyperbolic curve to give an accurate value of V_{max}. Linear transformations of the Michaelis–Menten equation are therefore preferred. The most popular of these is the Lineweaver–Burk equation, obtained by taking the reciprocal of the Michaelis–Menten equation:

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \tag{15.5}$$

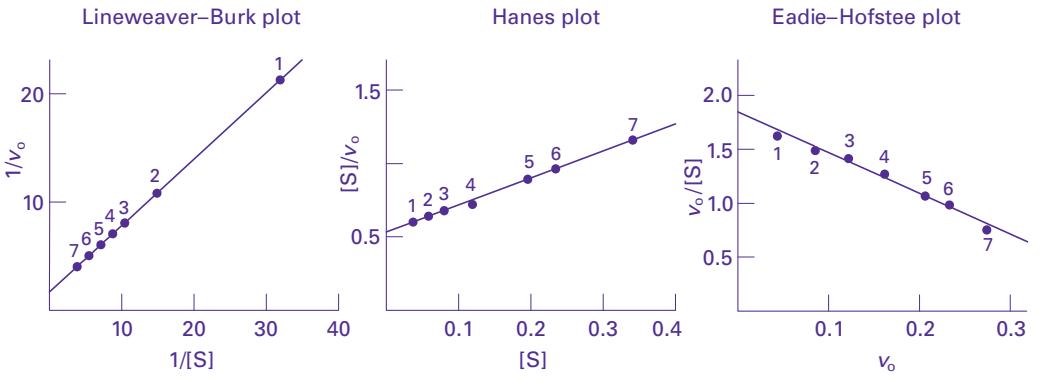


Fig. 15.6. Lineweaver–Burk, Hanes and Eadie–Hofstee plots for the same set of experimental data of the effect of substrate concentration, [S], on the initial rate, v_o of an enzyme-catalysed reaction.

A plot of $1/v_o$ against 1/[S] gives a straight line of slope K_m/V_{max}, with an intercept on the $1/v_o$ axis of $1/V_{max}$ and an intercept on the 1/[S] axis of $-1/K_m$. Alternative plots are based on the Hanes equation:

$$\frac{[S]}{v_o} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \tag{15.6}$$

so that $[S]/v_o$ is plotted against [S], and on the Eadie–Hofstee equation:

$$\frac{v_o}{[S]} = \frac{V_{max}}{K_m} - \frac{v_o}{K_m} \tag{15.7}$$

so that $v_o/[S]$ is plotted against v_o. The relative merits of the Lineweaver–Burk, Hanes and Eadie–Hofstee equations for the determination of K_m and V_{max} are illustrated in Fig. 15.6 using the same set of experimental values of v_o for a series of substrate concentrations (for further details, see Example 2).

It can be seen that the Lineweaver–Burk equation gives an unequal distribution of points and greater emphasis to the points at low substrate concentration that are subject to the greatest experimental error, whilst the Eadie–Hofstee equation and the Hanes equation give a better distribution of points. In the case of the Hanes plot, greater emphasis is placed on the experimental data at higher substrate concentrations and on balance it is the statistically preferred plot.

It is of course possible for two enzymes to have an affinity for the same substrate and, indeed, to have the same V_{max} value at a given enzyme concentration. If the two K_m values differ, however, the Michaelis–Menten plots will be different. This is shown in Fig. 15.7, which illustrates a concept that is important in the determination of the relative importance of branching in metabolic pathways. The same principle applies to the case where a given enzyme can act on two different substrates with different K_m values but the same or different V_{max} values. The reaction with the numerically smaller K_m value will be preferred at low substrate concentrations.

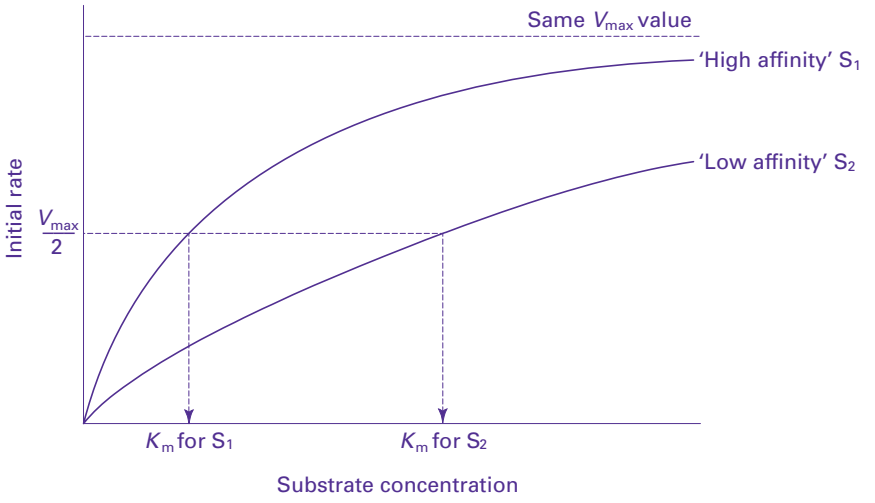


Fig. 15.7. The effect of the Michaelis constant, K_m, on the kinetic profile of an enzyme acting on two different substrates, S_1 and S_2. At low substrate concentrations the high affinity substrate will be the preferred substrate.

It is important to appreciate that, whilst K_m is a characteristic of an enzyme for its substrate and is independent of the amount of enzyme used for its experimental determination, this is not true of V_{max}. It has no absolute value but varies with the amount of enzyme used. This is illustrated in Fig. 15.5 and is discussed further in the Example 2. A valuable catalytic constant in addition to K_m and V_{max} is the turnover number, k_{cat}, defined as:

$$k_{cat} = \frac{V_{max}}{[E_t]} \tag{15.8}$$

where $[E_t]$ is the total concentration of enzyme. The turnover number is the maximum number of moles of substrate that can be converted to product per mole of enzyme in unit time. It has units of reciprocal time in seconds. Its values range from 1 to $10^7\,s^{-1}$. Catalase has a turnover number of $4 \times 10^7\,s^{-1}$ and is one of the most efficient enzymes known. The catalytic potential of high turnover numbers can be realised only at high (saturating) substrate concentrations and this is seldom achieved under normal cellular conditions. An alternative constant, termed the specificity constant, defined as k_{cat}/K_m, is a measure of how efficiently an enzyme converts substrate to product at low substrate concentrations. It has units of $(\text{mol dm}^{-3})^{-1}\,s^{-1}$.

For a substrate to be converted to product, molecules of the substrate and of the enzyme must first collide by random diffusion and then combine in the correct orientation. Diffusion and collision have a theoretical limiting value of about $10^9\,(\text{mol dm}^{-3})^{-1}\,s^{-1}$ and yet many enzymes, including acetylcholine esterase, carbonic anhydrase, catalase, β-lactamase and triosephosphate isomerase, have specificity constants approaching this value, indicating that they have evolved to almost maximum kinetic efficiency. Since specificity constants

Example 2 PRACTICAL ENZYME KINETICS

Question

The enzyme α-D-glucosidase isolated from *Saccharomyces cerevisiae* was studied using the synthetic substrate *p*-nitrophenyl-α-D-glucopyranoside (PNPG), which is hydrolysed to release *p*-nitrophenol, which is yellow in alkaline solution (for further details, see Section 15.2.2). A 3 mM solution of PNPG was prepared and portions used to study the effect of substrate concentration on initial rate using a fixed volume of enzyme preparation. The total volume of each assay mixture was 10 cm^3. A 1 cm^3 sample of the reaction mixture was withdrawn after 2 min, placed in 4 cm^3 borate buffer, pH 9.0, to stop the reaction and develop the yellow colour. The change in absorbance at 400 nm was determined and used as a measure of the initial rate. The following results were obtained:

PNPG (cm^3)	0.1	0.2	0.3	0.4	0.6	0.8	1.2
Initial rate	0.055	0.094	0.130	0.157	0.196	0.230	0.270

What kinetic constants can be obtained from this data?

Answer

Subject to the calculation of the molar concentration of PNPG in each reaction mixture, it is possible to construct Lineweaver–Burk, Hanes and Eadie–Hofstee plots to obtain the values of K_m and V_{max}. The fact that a 1 cm^3 sample of the reaction mixture was used to measure the initial rate is not relevant to the calculation of [S]. Lineweaver–Burk, Hanes and Eadie–Hofstee plots derived from this data are shown in Fig. 15.6 in which v_o measurements are expressed simply as the increase in absorption at 400 nm. The plots give K_m values of approximately 0.2 mM and V_{max} values of approximately 0.4.

As pointed out in Section 15.3.1, V_{max} values can be expressed in a variety of units and their experimental value is dependent on a number of variables, particularly the concentration of enzyme. For comparative reasons, V_{max} is best expressed in terms of the number of moles of product formed in unit time. To do this, it is necessary to convert absorbance units to amount of product by means of a Beer–Lambert law plot. Data for such a plot in this experiment is given in Table A.

Table A

[PNP] (μM)	2.0	4.0	6.0	8.0	12.0	16.0	24.0
Absorbance (400 nm)	0.065	0.118	0.17	0.23	0.34	0.45	0.65

A plot of these data confirms that the Beer–Lambert law is held and enables the amount of product to be calculated. From this, v_o values in units of μmol min^{-1} can be calculated. The data for the three linear plots are presented in Table B.

Table B

[S] (mM)	0.03	0.06	0.09	0.12	0.18	0.24	0.36
v_o (μmol min^{-1})	0.054	0.096	0.138	0.168	0.210	0.251	0.294
$1/[S]\ (mM)^{-1}$	33.33	16.67	11.11	8.33	5.55	4.17	2.78
$1/v_o\ (\mu mol\ min^{-1})^{-1}$	18.52	10.42	7.25	5.95	4.76	3.98	3.40
$v_o/[S] \times 10^3\ (dm^3\ min^{-1})$	1.8	1.6	1.53	1.40	1.17	1.05	0.82
$[S]/v_o \times 10^{-3}\ (min\ dm^{-3})$	0.56	0.63	0.65	0.71	0.85	0.95	1.22

Example 2 (*Cont.*)

Data derived from the three linear plots are presented in Table C.

Table C

Plot	Regression coefficient	Slope	Intercept	K_m (mM)	V_{max} (μmol min^{-1})
Lineweaver–Burk	0.9997	0.499	1.91	0.26	0.52
Hanes	0.9970	1.990	0.489	0.25	0.50
Eadie–Hofstee	0.9930	−3.995	2.030	0.25	0.51

The agreement between the three plots for the values of K_m and V_{max} was good but the quality of the fitted regression line for the Lineweaver–Burk plot was noticeably better. However, the distribution of the experimental points along the line is the poorest for this plot (Fig. 15.6). The value for V_{max} indicates the amount of product released per minute, but of course this is for the chosen amount of enzyme and is for 10 cm^3 of reaction mixture. For V_{max} to have any absolute value, the amount of enzyme and the volume of reaction mixture have to be taken into account. The volume can be adjusted to 1 dm^3 giving a V_{max} of 51 μmol min^{-1}dm^{-3}, but it is only possible to correct for the enzyme amount if it was pure and of a known amount in molar terms. The enzyme is known to have a molecular mass of 68 kDa so, if there were 3 μg of pure enzyme in each 10 cm^3 reaction mixture, its molar concentration would be 4.4×10^{-3} μM. This allows the value of the turnover number k_{cat} to be calculated (see equation 15.8):

$$k_{cat} = V_{max}/[E_t] = 51\,\mu\text{mol min}^{-1}\text{dm}^{-3}/4.4 \times 10^{-3}\,\mu\text{mol dm}^{-3}$$
$$= 11 \times 10^3\,\text{min}^{-1} \text{ or } 1.8 \times 10^2\,\text{s}^{-1}$$

k_{cat} is a measure of the number of molecules of substrate (PNPG in this case) converted to product per second by the enzyme under the defined experimental conditions. The value of 1.8×10^2 is in the mid-range for the majority of enzymes. It is also possible to calculate the specificity constant that is a measure of the efficiency with which the enzyme converts substrate to product at low (K_m) substrate concentrations:

$$k_{cat}/K_m = 1.8 \times 10^2\,\text{s}^{-1}/0.25\,\text{mM} = 7.2 \times 10^3\,(\text{mM})^{-1}\text{s}^{-1} \text{ or } 7.2 \times 10^5\,(\text{mM})^{-1}\text{s}^{-1}$$

Note that the units of the specificity constant are that of a second-order rate constant, effectively for the conversion of E + S to E + P. Its value in this case is typical of many enzymes.

are a ratio of two other constants, enzymes with similar specificity constants can have widely different K_m values. As an example, catalase has a specificity constant of 4×10^7 (mol dm^{-3})$^{-1}$s^{-1} with a K_m of 1.1 M (very high), whilst fumarase has a specificity constant of 3.6×10^7(M)$^{-1}$s^{-1} with a K_m of 2.5×10^{-5}M (very low). Multienzyme complexes overcome some of the diffusion and collision limitations to specificity constants. The product of one reaction is passed directly by a process called channelling to the active site of the next enzyme in the pathway

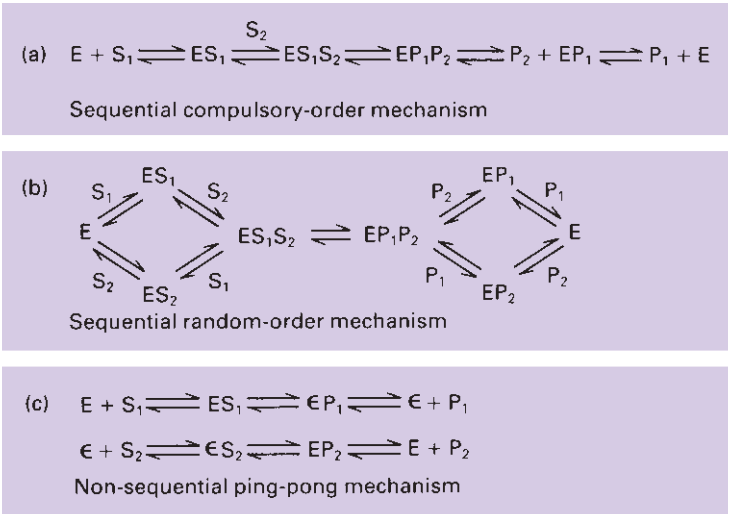


Fig. 15.8. Possible reaction mechanisms for bisubstrate reactions.

as a consequence of its juxtaposition in the complex, thereby eliminating diffusion limitations (Section 15.4.7).

15.3.2 Bisubstrate enzyme reactions

Bisubstrate reactions (Fig. 15.8) such as those catalysed by the transferases, kinases and dehydrogenases, in which two substrates S_1 and S_2 are converted to two products P_1 and P_2 (two substrate, two product, bi-bi, reactions), are inherently more complicated than monosubstrate reactions in that they may be as follows:

- *Sequential:* In this case both substrates bind at specific regions within the enzyme active site to give a ternary complex before the products are formed. Sequential reactions may be either compulsory ordered, in which case the two substrates bind in a definite sequence, or random ordered, in which case either substrate can bind first.
- *Non-sequential:* In this case one product is released before the second substrate is bound.

A compulsory-order and a random-order ternary complex mechanism both give non-parallel Lineweaver–Burk double reciprocal plots with a progressively smaller intercept on the $1/v_0$ axis as the concentration of fixed second substrate is increased (Fig. 15.8b).

An example of the non-sequential mechanism is a ping-pong reaction, which proceeds via a modified form of the enzyme (ε) that may take the form of an acylated enzyme. A ping-pong mechanism is indicated, but not confirmed, by a series of parallel lines in Lineweaver–Burk double reciprocal plots when the variation of initial rate with increasing concentration of one substrate is investigated in the

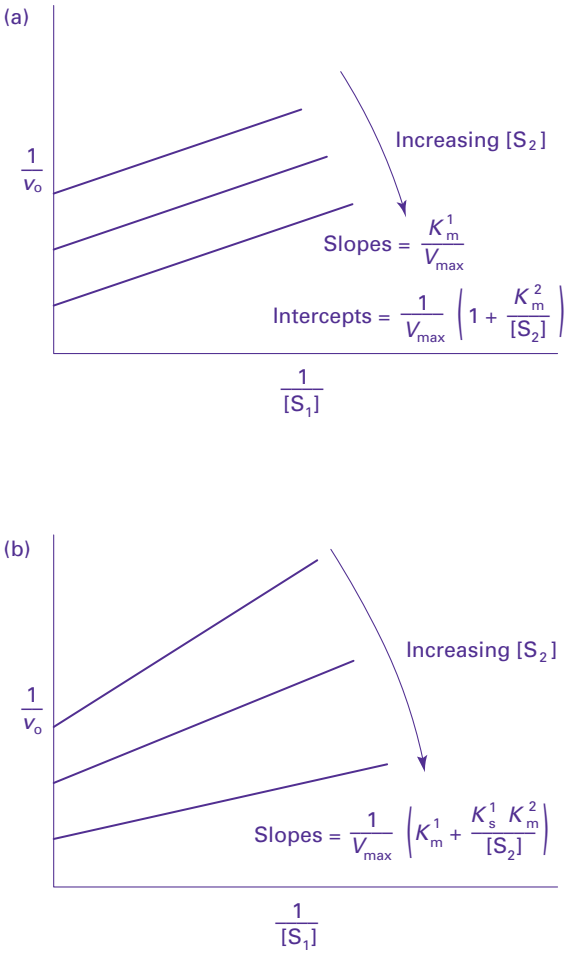


Fig. 15.9. Lineweaver–Burk plots for bisubstrate reactions. (a) For a ping-pong bi-bi mechanism, a series of parallel plots is obtained. (b) For a random or ordered bi-bi mechanism a series of plots that converge to the left of the *x*-axis is obtained. K_m^1 and K_m^2 are the Michaelis constants for substrates S_1 and S_2, respectively. K_s^1 is the dissociation constant for ES_1.

presence of a series of fixed concentrations of the second substrate. Double reciprocal plots give a progressively smaller intercept on the 1/[S] axis as the concentration of second substrate is increased (Fig. 15.8a).

In these bisubstrate reactions, V_{max} is defined as the maximum initial rate when both substrates are saturating, and the K_m for a particular substrate as the concentration of that substrate which gives 0.5 V_{max} when the other substrate is saturating. To determine these K_m values, the initial velocity is studied as a function of the concentration of one substrate at a series of fixed second substrate concentrations. A double reciprocal plot is made for each second substrate concentration, giving a series of straight lines called primary plots (Fig. 15.9). A secondary plot is then made

Table 15.1 Patterns of product inhibition to distinguish sequential bisubstrate mechanisms for the conversion of two substrates, S_1 and S_2, to two products, P_1 and P_2

Mechanism	Product	S_1 variable	S_2 variable
Ordered bi-bi	P_1	Mixed	Mixed
	P_2	Competitive	Mixed
Random bi-bi	P_1	Competitive	Competitive
	P_2	Competitive	Competitive

of the $1/v_o$ intercepts of the primary Lineweaver–Burk plots against the reciprocal of the second (fixed) substrate. This gives a straight line, slope K_m (for the second substrate)/V_{max} and intercept $1/V_{max.}$ This study is then repeated reversing the roles of the two substrates. The principle of secondary plots is illustrated in Fig. 15.15.

The elucidation of the reaction mechanism associated with a particular bisubstrate reaction generally involves a study of the variation of the initial rate with the concentration of one substrate at a series of fixed concentrations of the second substrate, in the absence and presence of the two reaction products, and the application of a series of rules formulated by W. Cleland. Two of these rules are that:

- the intercept on the $1/v_o$ axis of double reciprocal plots is affected by an inhibitor only if it binds reversibly to an enzyme form other than that to which the variable substrate binds;
- the slope of double reciprocal plots is affected by an inhibitor that binds to the same enzyme form as the variable substrate or to an enzyme form that is connected by a series of reversible steps to that to which the variable substrate binds.

The consequence of the first rule is that, if characteristic competitive inhibition (Section 15.3.7) behaviour is observed, the inhibitor and the substrate whose concentration is being varied bind at the same site. The consequence of the second rule is that, if characteristic uncompetitive inhibition (Section 15.3.7) is observed, there must be no reversible link between the inhibitor and the substrate whose concentration is being varied (Table 15.1).

Studies applying these rules have, for example, revealed that lactate dehydrogenase operates via a compulsory-order mechanism in which the NAD^+ binds first whereas phosphoglycerate mutase from yeast or mammalian sources (but not plant), which interconverts 2-phospho-D-glycerate (2PG) and 3-phospho-D-glycerate (3PG), operates via a ping-pong mechanism involving 2,3-bisphospho-D-glycerate (BPG) as a primer and a phosphorylated enzyme intermediate:

$$E + BPG \rightleftharpoons E\text{–}P + 2PG$$
$$E\text{-}P + 3PG \rightleftharpoons E + BPG$$

Net reaction:

$$3PG \rightleftharpoons 2PG$$

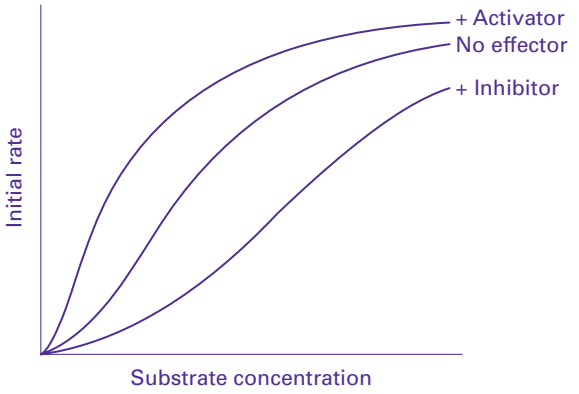


Fig. 15.10. Effect of activators and inhibitors on the sigmoidal kinetics of an enzyme subject to allosteric control.

15.3.3 Oligomeric enzyme reactions

A number of enzymes contain several protein subunits, which may be identical or different, and multiple catalytic sites. Such enzymes are said to be oligomeric and some do not display simple Michaelis–Menten kinetics, but give a sigmoidal relationship between initial rate and substrate concentration (Fig. 15.10). Such a curve is indicative of an allosteric enzyme, which is one whose catalytic activity is modified by conformational changes induced in the enzyme. In cases where these conformational changes are the result of the progressive binding of substrate molecules, the process is referred to as a homotropic effect. These conformational changes are initiated in one subunit but are transmitted to others, thereby altering the ease of binding of further substrate molecules. Progressive binding of the substrate molecules to the subunits is therefore said to be cooperative. This cooperativity may result in either increased (positive cooperativity) or decreased (negative cooperativity) activity towards the binding of further substrate molecules. Changes in catalytic activity towards the substrate may also be brought about by conformational changes induced by the binding of molecules other than the substrate at distinct allosteric binding sites on one or more subunit. Compounds that induce such changes are referred to as heterotropic effectors. They are commonly key metabolic intermediates such as ATP, ADP, AMP and P_i that, on binding to the allosteric site, change the conformation of the catalytic site. Heterotropic activators increase the catalytic activity of the enzyme, making the curve less sigmoidal and moving it to the left, whilst heterotropic inhibitors cause a decrease in activity, making the curve more sigmoidal and moving it to the right (Fig. 15.10). The diagnosis of cooperativity by use of the Lineweaver–Burk plot is shown in Fig. 15.14c. The operation of cooperative effects may be confirmed by a Hill plot, which is based on the equation:

$$\frac{\log v_o}{V_{max} - v_o} = h \log [S] + \log K \qquad (15.9)$$

where h is the Hill constant or coefficient, and K is an overall binding constant related to the individual binding constants for n sites.

The Hill constant, which is equal to the slope of the plot, is a measure of the cooperativity between the sites such that: if $h = 1$, binding is non-cooperative and normal Michaelis–Menten kinetics exist; if $h > 1$, binding is positively cooperative; and if $h < 1$, binding is negatively cooperative. At very low substrate concentrations that are insufficient to fill more than one site and at high concentrations at which most of the binding sites are occupied, the slopes of Hill plots tend to a value of 1. The Hill coefficient is therefore taken from the linear central portion of the plot. One of the problems with Hill plots is the difficulty of estimating V_{max} accurately. It is sometimes argued that h is numerically equal to the number of binding sites, n, for the substrate. This is an oversimplification and very often h is not an integer. For example, h for the binding of oxygen to haemoglobin, for which the number of binding sites is known to be four, is 2.6. In practice, h can be taken to be a minimum estimate of the number of interacting binding sites as well as a measure of the cooperativity.

The Michaelis constant K_m is not used with allosteric enzymes. Instead, the term $S_{0.5}$, which is the substrate concentration required to produce 50% saturation of the enzyme, is used. It is important to appreciate that sigmoidal kinetics do not confirm the operation of allosteric effects because sigmoidicity may be the consequence of the enzyme preparation containing more than one enzyme capable of acting on the substrate. It is easy to establish the presence of more than one enzyme, as there will be a discrepancy between the amount of substrate consumed and the expected amount of product produced.

Models of allosterism

Several models have been proposed to interpret allosteric regulation. They are all based on the assumption that the allosteric enzyme consists of a number of subunits (protomers) each of which can bind substrate and exist in two conformations referred to as the R (relaxed) and T (tense) states. It is assumed that the substrate binds more tightly to the R form. One of the most successful models, proposed by Jacques Monod, Jeffries Wyman and Jean-Pierre Changeux, is the symmetry model. It assumes that conformational change between the R and T states is highly coupled so that all subunits must exist in the same conformation. Thus binding of substrate to a T state protomer, causing it to change conformation to the R state, will automatically switch the other protomers to the R form, thereby enhancing reactivity (Fig. 15.11). The alternative induced-fit or sequential model of Daniel Koshland does not assume the tightly coupled concept and hence allows protomers to exist in different conformations but in such a way that binding to one protomer enhances the reactivity of others. Conformational changes in a protein can be studied by spectroscopic and sedimentation techniques but X-ray crystallography gives the most clear-cut evidence. Thus the enzyme aspartate carbamoyltransferase, which catalyses the first step in pyrimidine biosynthesis in *Escherichia coli*, has been shown by crystallography to display many of the characteristics of the symmetry model. In this case, CTP acts as an allosteric activator and ATP as an allosteric inhibitor.

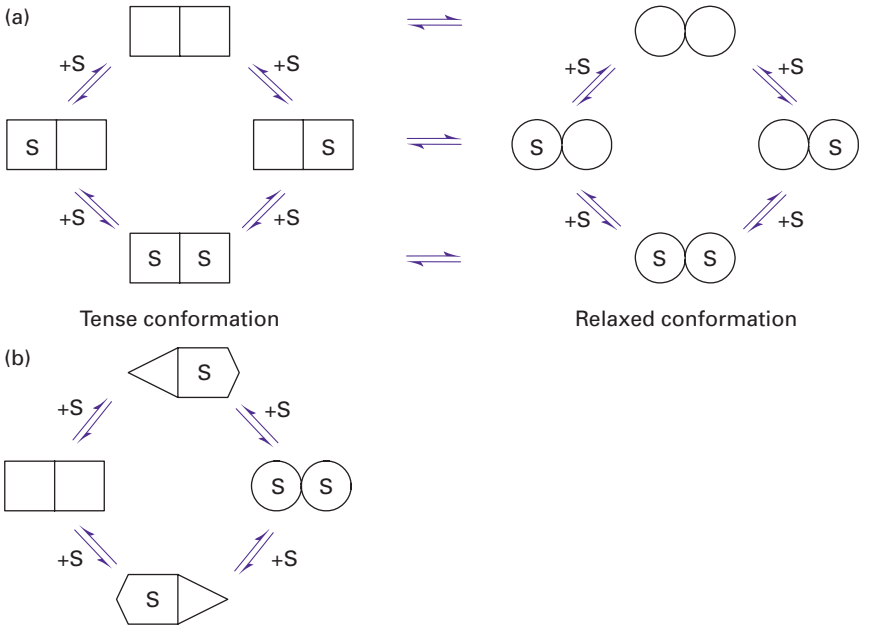


Fig. 15.11. Symmetry and sequential models of allosterism for a dimeric enzyme. (a) Symmetry model. The enzyme is assumed to exist in two conformations T and R, which differ in their affinity for the substrate. Conformational changes within each subunit (protomer) are assumed to be tightly coupled so that each can exist only in the same conformation. The binding of the substrate disturbs the equilibrium between the T and R states in favour of the R state, giving a concerted structure change such that the symmetry of the enzyme is preserved. Note that the model cannot explain how binding of a substrate molecule to one protomer decreases the affinity of the other protomer for the substrate, i.e. negative cooperativity. (b) Sequential model. Substrate binding to one protomer induces a change in its conformation and a cooperative interaction with the neighbouring protomer such that its reactivity towards substrate binding may be either enhanced (positive cooperativity) or decreased (negative cooperativity). The structure of individual protomers changes sequentially. Unlike the symmetry model, the sequential model does not preserve the symmetry of the enzyme during substrate binding.

An example of an enzyme displaying allosteric regulation is 6-phosphofructokinase (PFK), the key regulatory enzyme of glycolysis:

$$\text{D-fructose-6-phosphate} + \text{ATP} \xrightleftharpoons{\text{PFK}} \text{D-fructose 1,6-bisphosphate} + \text{ADP}$$

PFK is a tetramer in most organisms but exists as an octomer in yeasts. It can exist in two states consistent with the Koshland model (see below). Each subunit has a catalytic site and an allosteric inhibitor site for ATP. The catalytic site binds ATP in both the R and T states but the allosteric site binds only ATP in the T state. At low concentrations ATP is a substrate but at higher concentrations it acts as an inhibitor by binding to the T state and as a consequence reducing the affinity for the other substrate, fructose 6-phosphate, and reducing the rate of the glycolytic pathway. The enzyme is also inhibited by citrate and phosphoenol pyruvate but

activated by ADP, P_i, AMP and fructose 2,6-bisphosphate. These activators preferentially bind to the R state thus releasing the ATP inhibition. The balance between activation and inhibition is determined by the relative availability of these effectors. These effects are readily rationalised in terms of cellular energy demands and the associated need for the glycolytic pathway to be activated or inhibited. The control of PFK activity is discussed further in Section 15.5.2.

15.3.4 Effect of enzyme concentration

It can be shown that for monosubstrate enzymatic reactions that obey simple Michaelis–Menten kinetics:

$$v_o = \frac{k_{+2}[E][S]}{K_m + [S]}$$

and hence that

$$v_o = \frac{k_{+2}[E]}{K_m/[S] + 1} \tag{15.10}$$

Thus, when the substrate concentration is very large, equation 15.10 reduces to $v_o = k_{+2}[E]$, i.e. the initial rate is directly proportional to the enzyme concentration. This is the basis of the experimental determination of enzyme activity in a particular biological sample (Section 15.2). Fig. 15.3 illustrates the importance of the correct measurement of initial rate.

15.3.5 Effect of temperature

The initial rate of an enzyme reaction varies with temperature according to the Arrhenius equation:

$$\text{rate} = A\,e^{-E/RT} \tag{15.11}$$

where A is a constant known as the pre-exponential factor, which is related to the frequency with which molecules of the enzyme and substrate collide in the correct orientation to produce the enzyme–substrate complex, E is the activation energy (J mol^{-1}), R is the gas constant (8.2 J mol^{-1} K^{-1}), and T is the absolute temperature (K). Thus a plot of the natural logarithm of the initial rate (or better k_{cat}) against the reciprocal of the absolute temperature allows the value of E to be determined. Equation 15.11 explains the sensitivity of enzyme reactions to temperature as the relationship between reaction rate and absolute temperature is exponential. The rate of most enzyme reactions approximately doubles for every 10 deg.C rise in temperature (Q_{10} value). At a temperature characteristic of the enzyme, and generally in the region 40–70 °C, the enzyme is denatured and enzyme activity is lost. The activity displayed in this 40–70 °C temperature range depends partly upon the equilibration time before the reaction is commenced. The so-called optimum temperature, at which the enzyme appears to have maximum activity, therefore arises from a mixture of thermal stability, temperature coefficient and incubation time

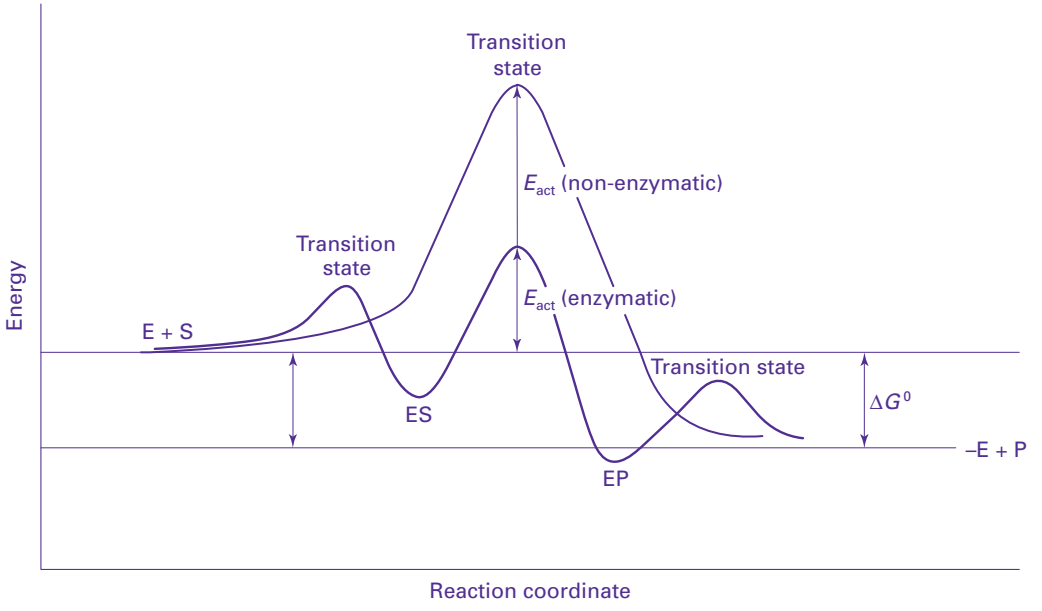


Fig. 15.12. Energy profile of a simple enzyme-catalysed reaction. The formation of ES and EP and the subsequent release of E + P proceeds via several transition states. The activation energy for the overall reaction is dictated by the initial free energy of E and S and the highest energy transition state. The non-enzyme-catalysed reaction proceeds via a higher energy transition state and hence the reaction has a higher activation energy than the enzyme-catalysed reaction.

and for this reason is not normally chosen for the study of enzyme activity. Enzyme assays are routinely carried out at 30 or 37 °C (Section 15.2). Interestingly, recent work with enzymes from mesophiles and thermophiles has indicated that some have a genuine temperature optimum in that above a certain temperature the enzyme becomes reversibly less active but not as a consequence of denaturation. The nature of the structural changes responsible for such observations has yet to be determined.

Enzymes work by facilitating the formation of the transition state, thereby decreasing the activation energy for the reaction relative to the non-enzyme catalysed reaction (Fig. 15.12). A decrease in the energy barrier of as little as 5.7 kJ mol^{-1}, equivalent in energy terms to a hydrogen bond, will result in a 10-fold increase in reaction rate. The energy barrier is, of course, lowered equally for both the forward and reverse reactions, so that the position of equilibrium is unchanged. As an extreme example of the efficiency of enzyme catalysis, the enzyme catalase decomposes hydrogen peroxide 10^{14} times faster than does the uncatalysed reaction! Fig. 15.12 shows a simple energy profile for the conversion of a substrate to products as a function of the reaction coordinate that measures the time-related progress of the reaction. The number of energy barriers in the profile will depend upon the number of kinetically important stages in the reactions. For the majority of enzyme-catalysed reactions the major energy barrier, which

dictates the activation energy for the reaction and hence its rate, is the formation of one or more transition states, in which covalent bonds are being made and broken and which cannot be isolated. An example is shown in Figure 15.17. However, for a few enzymes, notably ATP synthase, the energy-requiring step is the initial binding of the substrate(s) and the subsequent release of the product(s).

Proof that enzymes function by facilitating the creation of the transition state has come from the development of transition state analogues, which mimic the structure of the transition state. For example, the transition state for the esterase hydrolysis of carboxylic acid esters is a tetrahedral intermediate that is mimicked by a phosphonate ester with similar substituents (Fig. 15.13). If a monoclonal antibody is raised to an antigen consisting of the phosphonate ester as the hapten (Section 7.2.2), one of the antibodies raised will be complementary to the tetrahedral phosphonate ester intermediate and will effectively behave as the active site of the esterase. Proof of this ability was confirmed by the ability of the antibody to convert the ester to products in the absence of esterase at an enhanced rate (by factor of up to 10^5) relative to the uncatalysed reaction. The success of these studies lay in using the optimum structures for R and R′. Antibodies with this ability to promote catalysis have been called catalytic antibodies or simply abzymes (*a*nti*b*odies as en*z*y*me*s) or mabzymes (*m*onoclonal *a*nti*b*odies as en*z*y*me*s).

The thermodynamic constants ΔG^0, ΔH^0 and ΔS^0 for the binding of substrate to the enzyme can be calculated from a knowledge of the binding constant, K_a ($= 1/K_s$). ΔG^0 can be obtained from the equation:

$$\Delta G^0 = -RT \ln K_a \tag{15.12}$$

If K_a is measured as a function of temperature, a plot of $\ln K_a$ versus $1/T$, known as the van't Hoff plot, will give a straight line of slope $-\Delta H^0/R$ with intercept on the y-axis of $\Delta S^0/R$, the relevant equation being:

$$\ln K_a = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT} \tag{15.13}$$

Recent research has indicated that a small number of enzymes may operate by a mechanism that does not rely on the formation of a transition state. Work with the enzyme methylamine dehydrogenase, which promotes the cleavage of a C—H bond, has shown that the reaction is independent of temperature and hence is inconsistent with transition state theory. The observation is explained in terms of enzyme-catalysed quantum tunnelling. Under this mechanism, rather than overcoming the potential energy barrier the reaction proceeds through (hence 'tunnelling') the barrier at an energy level near to that of the ground state of the reactants. Concerted enzyme and substrate vibrations are coupled in such a way as to reduce the width and height of the potential energy barrier and facilitate the cleavage of the C—H bond by the process of quantum mechanical tunnelling. This phenomenon is known to occur with some chemical reactions but only at low temperatures. The fine detail of precisely how enzymes promote this process remains to be elucidated.

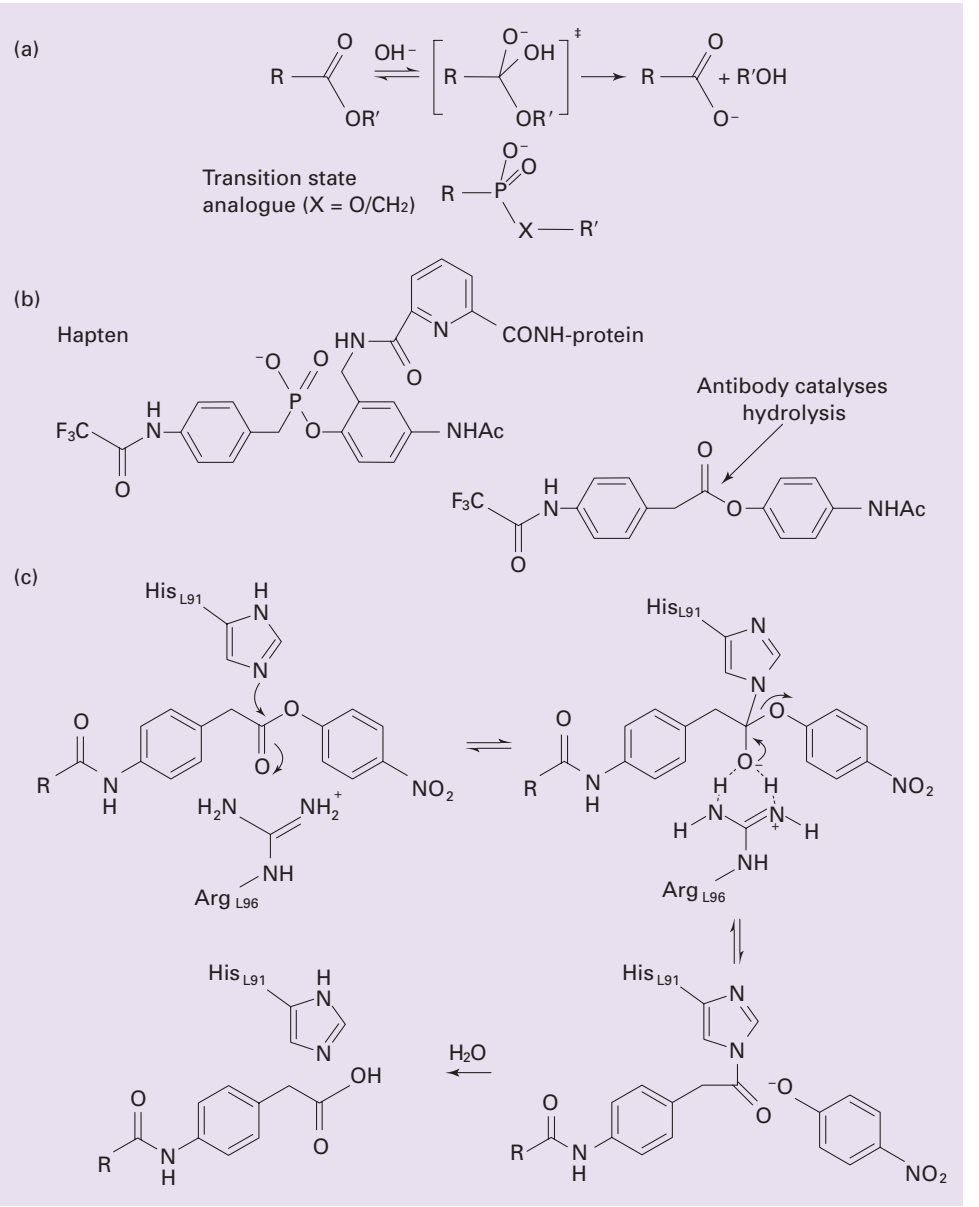


Fig. 15.13. Catalytic antibodies for ester hydrolysis. (a) The catalytic mechanism for ester hydrolysis involves a tetrahedral transition state. (b) The catalytic antibody is based on a hapten containing a tetrahedral organophosphorus compound resembling the ester transition state. The antibody raised against the hapten will contain a structural component complementary to the hapten and hence capable of binding an appropriate ester and facilitating the formation of the transition state. (c) The antibody 43C9 has been shown to promote ester hydrolysis by an acid-catalysed mechanism similar in principle to that of ribonuclease A (see Fig 15.17). (Reproduced from T. Bugg (2004), *An Introduction to Enzyme and Coenzyme Chemistry*, 2nd edn, by permission of Blackwell Science Limited.)

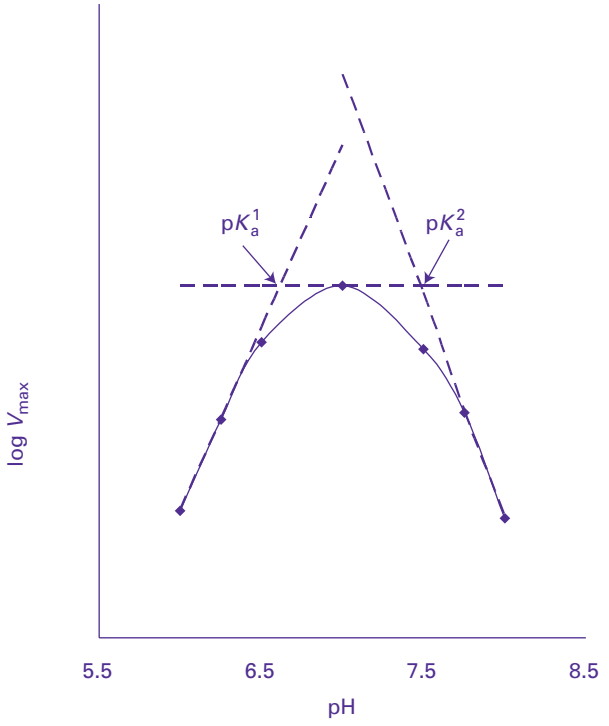


Fig. 15.14. The effect of pH on V_{max} of an enzyme-catalysed reaction involving two ionisable groups in the active site of the enzyme. The construction of tangents to the experimental line allows the pK_a values of the ionisable groups to be estimated.

15.3.6 Effect of pH

The state of ionisation of amino acid residues in the catalytic site of an enzyme is pH dependent. Since catalytic activity relies on a specific state of ionisation of these residues, enzyme activity is also pH dependent. As a consequence, plots of log K_m and log V_{max} (or better, k_{cat}) against pH are either bell shaped (indicating two important ionisable amino acid residues in the active site), giving a narrow pH optimum, or have a plateau (one important ionisable amino acid residue in the active site). In either case, the enzyme is generally studied at a pH at which its activity is maximal. By studying the variation of log K_m and log V_{max} with pH, it is possible to identify the pK_a values of key amino acid residues involved in the binding and catalytic processes (Fig. 15.14).

15.3.7 Effect of enzyme inhibitors

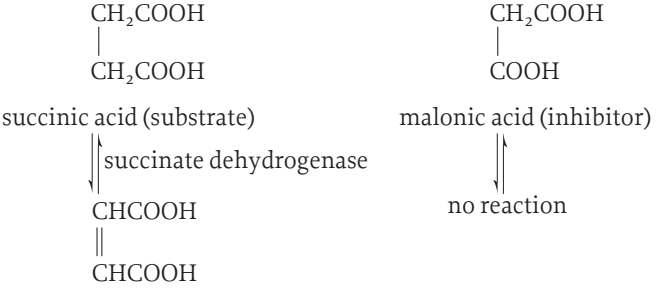
Irreversible inhibition

An enzyme inhibitor binds to an enzyme in such a way as to reduce the ability of the enzyme to either bind substrate and/or convert it to product. Irreversible inhibitors, such as the organophosphorus and organomercury compounds,

cyanide, carbon monoxide and hydrogen sulphide, combine with the enzyme to form a covalent bond. The extent of their inhibition of the enzyme is dependent upon the reaction rate constant (and hence time) for covalent bond formation and upon the amount of inhibitor present. The effect of irreversible inhibitors, which cannot be removed by simple physical techniques such as dialysis, is to reduce the amount of enzyme available for reaction. The inhibition involves reactions with a functional group, such as hydroxyl or sulphydryl, or with a metal atom in the active site or a distinct allosteric site. Thus the organophosphorus compound, diisopropylphosphofluoridate, reacts with a serine group in the active site of esterases such as acetylcholinesterase, whilst the organomercury compound *p*-hydroxymercuribenzoate reacts with a cysteine group, in both cases resulting in covalent bond formation and enzyme inhibition. Such inhibitors are valuable in the study of enzyme active sites (Section 15.4.2).

Competitive reversible inhibition

Reversible inhibitors combine non-covalently with the enzyme and can therefore be readily removed by dialysis. Competitive reversible inhibitors combine at the same site as the substrate and must therefore be structurally related to the substrate. An example is the inhibition of succinate dehydrogenase by malonate:



All types of reversible inhibitors are characterised by their dissociation constant K_i, called the inhibitor constant, which may relate to the dissociation of EI (K_{EI}) or of ESI (K_{ESI}), where I is the inhibitor. For competitive inhibition the following two equations can be written:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$E + I \rightleftharpoons EI \rightarrow \text{no reaction}$$

Since the binding of both substrate and inhibitor involves the same site, the effect of a competitive reversible inhibitor can be overcome by increasing the substrate concentration. The result is that V_{max} is unaltered but the concentration of substrate required to achieve it is increased so that when $v_o = 0.5\,V_{max}$ then:

$$[S] = \frac{K_m(1 + [I])}{K_i} \tag{15.14}$$

where [I] is the concentration of inhibitor.

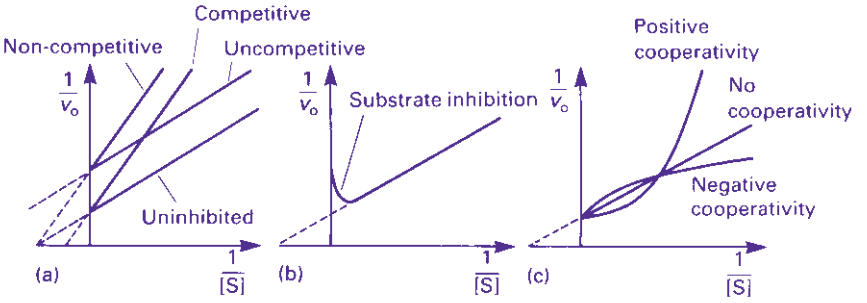


Fig. 15.15. Lineweaver–Burk plots showing (a) the effects of three types of reversible inhibitor, (b) substrate inhibition, and (c) homotropic cooperativity.

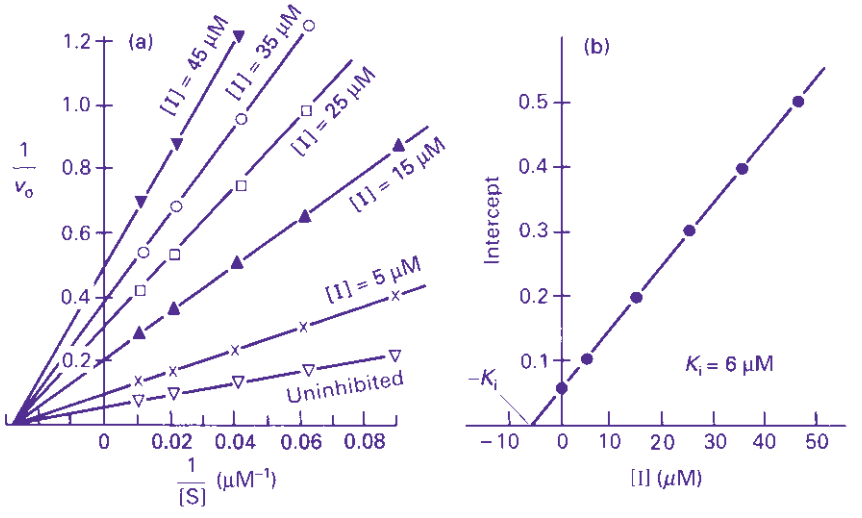


Fig. 15.16. (a) Primary Lineweaver–Burk plots showing the effect of a simple linear non-competitive inhibitor at a series of concentrations and (b) the corresponding secondary plot that enables the inhibitor constant K_i to be calculated.

It can be seen from equation 15.14 that K_i is equal to the concentration of inhibitor that apparently doubles the value of K_m. With this type of inhibition, K_i is equal to K_{EI} whilst K_{ESI} is infinite because no ESI is formed. In the presence of a competitive inhibitor, the Lineweaver–Burk equation (15.5) becomes:

$$\frac{1}{v_o} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} \frac{(1 + [I])}{(K_i)} + \frac{1}{V_{max}} \qquad (15.15)$$

Application of this equation allows the diagnosis of competitive inhibition (Fig. 15.15a). The numerical value of K_i can be calculated from Lineweaver–Burk plots for the uninhibited and inhibited reactions. In practice, however, a more accurate value is obtained from a secondary plot (Fig. 15.16). The reaction is carried out for a range of substrate concentrations in the presence of a series of

fixed inhibitor concentrations and a Lineweaver–Burk plot for each inhibitor concentration constructed. Secondary plots of the slope of the primary plot against the inhibitor concentration or of the apparent K_m, (K'_m) (which is equal to K_m {(1 + [I])/K_i} and which can be calculated from the reciprocal of the negative intercept on the I/[S] axis) against inhibitor concentration, will both have intercepts on the inhibitor concentration axis, of $-K_i$. Sometimes it is possible for two molecules of inhibitor to bind at the active site. In these cases, although all the primary double reciprocal plots are linear, the secondary plot is parabolic. This is referred to as parabolic competitive inhibition to distinguish it from normal linear competitive inhibition.

Non-competitive reversible inhibition

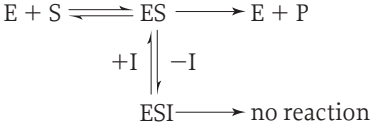
A non-competitive reversible inhibitor combines at a site distinct from that for the substrate. Whilst the substrate can still bind to its catalytic site, resulting in the formation of a ternary complex ESI, the complex is unable to convert the substrate to product and is referred to as a dead-end complex. Since this inhibition involves a site distinct from the catalytic site, the inhibition cannot be overcome by increasing the substrate concentration. The consequence is that V_{max}, but not K_m, is reduced because the inhibitor does not affect the binding of substrate but it does reduce the amount of free ES that can proceed to the formation of product. With this type of inhibition, K_{EI} and K_{ESI} are identical and K_i is numerically equal to both of them. In this case the Lineweaver–Burk equation (15.5) becomes:

$$\frac{1}{v_o} = \frac{K_{m-}}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \left(\frac{1 + [I]}{K_i} \right) \tag{15.16}$$

Once non-competitive inhibition has been diagnosed (Fig. 15.15a), the K_i value is best obtained from a secondary plot of either the slope of the primary plot or of $1/V'_{max}$ (which is equal to the intercept on the $1/v_o$ axis) against inhibitor concentration. Both secondary plots will have an intercept of $-K_i$ on the inhibitor concentration axis (Fig. 15.16).

Uncompetitive reversible inhibition

An uncompetitive reversible inhibitor can bind only to the ES complex and not to the free enzyme, so that inhibitor binding must be either at a site created by a conformational change induced by the binding of the substrate to the catalytic site or directly to the substrate molecule. The resulting ternary complex, ESI, is also a dead-end complex.



As with non-competitive inhibition, the effect cannot be overcome by increasing the substrate concentration, but in this case both K_m and V_{max} are reduced by a factor of (1 + [I])/K_i. An inhibitor concentration equal to K_i will therefore halve

the values of both K_m and V_{max}. With this type of inhibitor, K_{EI} is infinite because the inhibitor cannot bind to the free enzyme so K_i is equal to K_{ESI}. The Lineweaver–Burk equation (15.5) therefore becomes:

$$\frac{1}{v_0} = \left(\frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}\right)\left(\frac{1+[I]}{K_i}\right) \tag{15.17}$$

The value of K_i is best obtained from a secondary plot of either $1/V'_{max}$ or I/K'_m (which is equal to the intercept on the I/[S] axis) against inhibitor concentration. Both secondary plots will have an intercept of $-K_i$ on the inhibitor concentration axis.

Mixed reversible inhibition

For some inhibitors either the ESI complex has some catalytic activity or the K_{EI} and K_{ESI} values are neither equal nor infinite. In such case so-called mixed inhibition kinetics are obtained. Mixed inhibition is characterised by a linear Lineweaver–Burk plot that does not fit any of the patterns shown in Fig. 15.15a. The plots for the uninhibited and inhibited reactions may intersect either above or below the 1/[S] axis. The associated K_i can be obtained from a secondary plot of the slope either of the primary plot or of $1/V_{max}$ for the primary plots against inhibitor concentration. In both cases the intercept on the inhibitor concentration axis is $-K_i$. Non-competitive inhibition may be regarded as a special case of mixed inhibition.

Substrate inhibition

A number of enzymes at high substrate concentration display substrate inhibition characterised by a decrease in initial rate with increased substrate concentration. The graphical diagnosis of this situation is shown in Fig. 15.15b. It is explicable in terms of the substrate acting as an uncompetitive inhibitor and forming a dead-end complex.

End-product inhibition

First enzymes in an unbranched metabolic pathway are commonly regulated by end-product inhibition. Here the final product of the pathway acts as an inhibitor of the first enzyme thus switching off the whole pathway when the final product begins to accumulate. The inhibition of aspartate carbamyltransferase by CTP in the CTP biosynthetic pathway is an example of this form of regulation. In branched pathways, product inhibition usually operates on the first enzyme after the branch point.

Applications of enzyme inhibition

The study of the classification and mechanism of enzyme inhibition is of importance in a number of respects:

- it gives an insight into the mechanisms by which enzymes promote their catalytic activity (Section 15.4.2),
- it gives an understanding of the possible ways by which metabolic activity may be controlled *in vivo*,

Table 15.2 Examples of enzyme inhibitors as therapeutic agents

Inhibitor	Enzyme	Application
Zidovudine (AZT), didanosine (ddI)	Reverse transcriptase	HIV therapy
Ritonavir, saquinavir	HIV protease	HIV therapy
Sulphonamides	Dihydrofolate synthase	Bacterial infections
Trimethoprim, methotrexate	Dihydrofolate reductase	Bacterial infections
Organophosphorus compounds	Cholinesterase	Insecticides
Celastatin	Dehydropeptidase-1	Enhancement of antibacterial action
Iproniazide	Monoamine oxidase	Mood control
Disulfiram	Aldehyde dehydrogenase	Treatment of alcoholism
Allopurinol	Xanthine oxidase	Treatment of gout
Mevinolin	Hydroxymethylglutaryl CoA reductase	Inhibition of cholesterol synthesis
Aspirin	Cyclo-oxygenase (COX-1 and COX-2)	Inhibition of prostaglandin and thromboxane synthesis

HIV, human immunodeficiency virus.

- it allows specific inhibitors to be synthesised and used as therapeutic agents to block key metabolic pathways underlying clinical conditions.

The use of enzyme inhibitors as therapeutic agents is of enormous commercial value. Examples of some such agents are given in Table 15.2. The HIV-1 protease inhibitors, such as ritonavir and saquinavir, were designed from knowledge of the active site of the aspartyl proteases of the pepsin class to which the HIV-1 protease belongs. The enzyme cleaves specific peptide bonds including -Phe-Pro- and -Tyr-Pro-. The two drugs were designed to resemble the tetrahedral transition state known to be involved in the catalytic mechanism of the enzyme. Both inhibitors contain bulky groups and a non-hydrolysable structure such that binding to the active site is possible but enzymatic cleavage is not, thus blocking the enzyme's action. The successful clinical use of the compounds relies on the fact that the drugs are sufficiently selective to be capable of binding to the HIV-1 protease but not the human aspartyl proteases, such as renin, involved in normal physiological functions.

15.4 ENZYME ACTIVE SITES AND CATALYTIC MECHANISMS

As previously pointed out, enzymes are characterised by their high specificity, catalytic activity and capacity for regulation. These properties must reflect the specific three-dimensional interaction between the enzyme and its substrate. A complete understanding of the way enzymes work must therefore include the elucidation of the mechanism underlying the binding of a substrate(s) to the enzyme catalytic site and the subsequent conversion of the substrate(s) to product(s). The mechanism must include details of the nature of the binding and

catalytic sites, the nature of the intermediate enzyme–substrate complex(es), and the associated electronic and stereochemical events that result in the formation of the product. A wide range of strategies and analytical techniques has been adopted to gain such an understanding and brief experimental details and the relative merits of each will now be considered.

15.4.1 X-ray crystallographic studies

X-ray crystallography is capable of giving, either directly or indirectly, decisive information about the mechanism of enzyme action. It requires the enzyme to be purified and obtained in crystalline form – itself a difficult challenge (Section 8.4.5). X-ray diffraction patterns enable the position of each amino acid in the protein to be located and the details deduced of how the substrate binds and undergoes reaction. Such deductions are facilitated by the study of crystals grown in the presence and absence of the substrate, competitive inhibitor or effector molecules. The limitation of the technique is that X-ray diffraction provides details of the enzyme in a static, stable state; yet proteins are known to be highly flexible and dynamic in solution and capable of existing in multiple microconformations. Fortunately, all the evidence to date confirms the applicability of the X-ray data to the aqueous *in vivo* environment. Classic examples of the power of this approach come from studies of lysozyme, ribonuclease, hexokinase, a number of peptidases, phosphorylase and triosephosphate isomerase, all of which have revealed distinct binding clefts or pockets within the protein three-dimensional structure into which the substrate can fit, often in the process inducing a structural change or reorientation of specific amino acid residues to create the catalytic site. Such induced changes support the induced-fit theory of active sites rather than the earlier lock–key hypothesis, which visualised the binding site as being a permanent, integral feature of the enzyme. The juxtaposition of specific amino acid residues, within the induced site, to particular covalent bonds in the substrate molecule commonly favours the establishment of hydrogen bonding or electrostatic attractions between the enzyme and the substrate and the formation of the energetically favourable transition state required for reaction. Amino acid residues commonly found in active sites are therefore those with side-chains containing key functional groups such as those found in the side-chains of aspartic acid, glutamic acid, serine, cysteine, histidine, lysine and arginine.

As knowledge of protein structures and catalytic mechanisms has increased, computer programs have become available that enable the chemical and stereochemical conformations of the substrate(s) to be modelled and a prediction made of the three-dimensional structure of the enzyme that promotes the formation of product(s). This approach is now widely used in the pharmaceutical industry to identify 'lead' compounds for the development of new drugs.

15.4.2 Irreversible inhibitor and affinity label studies

Irreversible inhibitors act by forming a covalent bond with the enzyme (Section 15.3.7). By locating the site of the binding of the inhibitor, information can often

be obtained about the identity of specific amino acids in the binding site. Thus cyanide binds to metal atoms that are important for the activity of some enzymes, whilst the organomercury compounds and iodoacetate react with sulphydryl groups of cysteine residues. The organophosphorus compounds, such as diisopropylphospho-fluoridate, are powerful inhibitors of acetylcholinesterase and serine proteases by virtue of the covalent bond they form with a serine hydroxyl group in the active site. This specific labelling of amino acids in the active site can be exploited by using an analogue of the natural substrate that contains a reactive group that will form a covalent bond with the enzyme. An example is the use of bromoglycerone phosphate to inhibit triosephosphate isomerase:

$$\underset{\text{3-bromoglycerone phosphate}}{O_3^{2-}POCH_2COCH_2Br} + \underset{\text{triosephosphate isomerase}}{\text{Enzyme-OH}} \longrightarrow \underset{\text{alkylated enzyme}}{\text{Enz-O-}CH_2COCH_2OPO_3^{2-}}$$

A development of this approach is the use of photoaffinity labels that structurally resemble the substrate but which contain a functional group, such as azo (-N=N-), which on exposure to light is converted to a reactive functional group, such as a carbene or nitrene, which forms a covalent bond with a neighbouring functional group in the active site. It is common practice to tag the inhibitor or photoaffinity label with a radioisotope so that its location in the enzyme protein can easily be established experimentally.

15.4.3 Kinetic studies

Kinetic studies using a range of substrates and/or competitive inhibitors and the determination of the associated K_m, k_{cat} and K_i values allow correlations to be drawn between molecular structure and kinetic constants and hence deductions to be made about the structure of the active site. In the case of bisubstrate reactions, information about the reaction mechanism and substrate binding sequence can be also be deduced (Section 15.3.2). Further information about the structure of the active site can be gained by studying the influence of pH on the kinetic constants. Specifically, the effect of pH on K_m (i.e. on binding of E to S) and on V_{max} or k_{cat} (i.e. conversion of ES to products) is studied. Plots are then made of the variation of log K_m with pH and of log V_{max} or log k_{cat} with pH. The intersection of tangents drawn to the curves gives an indication of the pK_a values of ionisable groups involved in the active site (Fig. 15.14). These are then compared with the pK_a values of the ionisable groups known to be in proteins. For example, pH sensitivity around the range 6–8 could reflect the importance of one or more imidazole side-chains of a histidine residue in the active site because of its known pK_a in this range.

15.4.4 Isotope-exchange studies

The replacement of the natural isotope of an atom in the substrate by a different isotope of the same element and the study of the impact of the isotope replacement

on the observed rate of enzymatic reaction and its associated stereoselectivity often enable deductions to be made about the mechanism of the reaction. Two examples illustrate the principle. First, alcohol dehydrogenase (AD), which oxidises ethanol to ethanal using NAD^+:

$$\underset{\text{ethanol}}{CH_3CH_2OH} + NAD^+ \overset{AD}{\rightleftharpoons} \underset{\text{ethanal}}{CH_3CHO} + NADH + H^+$$

The two hydrogen atoms on the methylene ($-CH_2$) group of ethanol are chemically indistinguishable, but if one is replaced by a deuterium or tritium atom the carbon atom becomes a chiral centre and the resulting molecule can be identified as either *R* or *S* configuration according to the Cahn–Ingold–Prelog rule for defining the stereochemistry of asymmetric centres. Studies have shown that alcohol dehydrogenase exclusively removes the hydrogen atom in the proR configuration, i.e. (*R*) CH_3CHDOH always loses the D isotope in its conversion to ethanal but (*S*)-CH_3CHDOH retains it. Such a finding can be interpreted only in terms of the specific orientation of the ethanol molecule at the binding site such that the two hydrogen atoms are effectively not equivalent. All dehydrogenases have been shown to display this type of stereospecificity and can be classified as either A-side dehydrogenases (e.g. alcohol dehydrogenase, lactate dehydrogenase, malate dehydrogenase) or B-side dehydrogenases (e.g. glycerol-3-phosphate dehydrogenase, glucose dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase). Interestingly, the class type is independent of the hydrogen acceptor being NAD^+ or $NADP^+$.

Secondly, esters may be hydrolysed by esterases that convert the ester to a mixture of acid and alcohol, simultaneously incorporating a molecule of water into the products:

$$\underset{\text{ester}}{RCOO^*R'} + H_2O \xrightarrow{\text{esterase}} \underset{\text{acid}}{RCOOH} + \underset{\text{alcohol}}{R'O^*H}$$

In this reaction the oxygen atom identified as O^* can be retained either in the acid or in the alcohol depending upon which side of the labelled oxygen atom the bond is broken, with water providing the second oxygen atom in the products. Labelling the oxygen atom in question as ^{18}O and studying, by mass spectrometry, its location after hydrolysis enables details to be drawn about the mechanism of the hydrolysis of the ester by the esterase. In practice, the labelled oxygen atom is found in the alcohol, which supports the view that the reaction mechanism involves initial attack by water, acting as a nucleophile, on the carbonyl carbon atom and the subsequent elimination of the $R'O^*$ group.

15.4.5 Spectrophotometric studies

NMR and Raman spectroscopy have both been used to deduce information about enzyme active sites. In the case of NMR, studies are confined to relatively small enzymes such as ribonuclease A. This single-chain protein contains four histidine

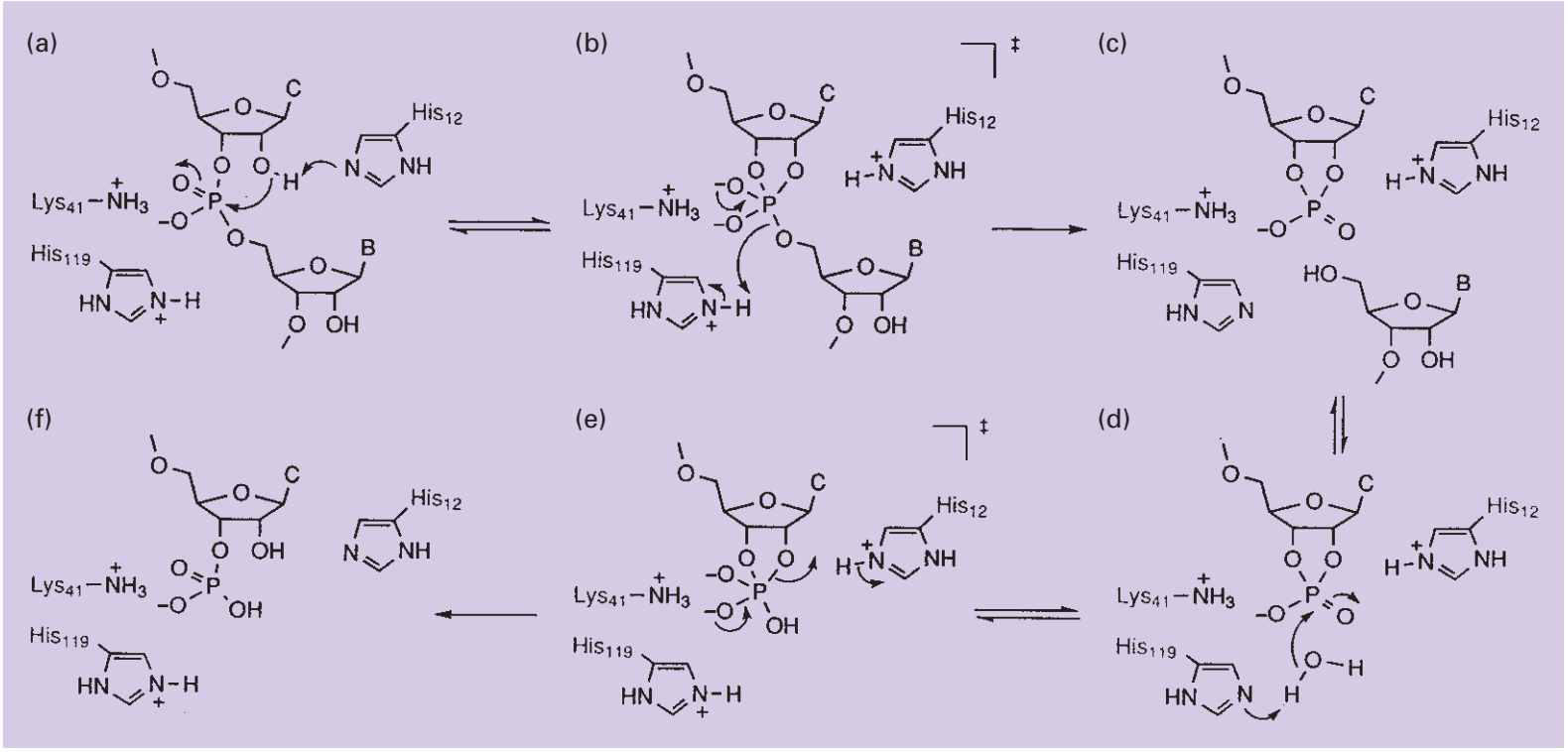


Fig. 15.17. Mechanism for the hydrolysis of RNA by ribonuclease A. (a) The RNA lies in a cleft of the ribonuclease such that the pyrimidine group, C, hydrogen bonds with Thr45. His12, acting as a base, removes a proton from the hydroxyl group, and the oxygen, acting as a nucleophile, attacks the phosphate group. (b) His119, acting as an acid, attacks the pentavalent phosphorus transition state cleaving the P-O link to the adjacent ribose group to form a cyclic phosphate diester on the first ribose group. (c) The 5′-OH product leaves the site. (d) A molecule of water attacks the cyclic phosphate diester that donates a proton to His119, acting as a base. (e) His12, acting as an acid, donates a proton to the second pentavalent phosphorus transition state to give the final 3′-phosphate product shown in (f). (Reproduced from T. Bugg (1997), *An Introduction to Enzyme and Coenzyme Chemistry*, by permission of Blackwell Science Limited.)

residues, two of which are implicated by pH/activity studies to be involved in enzymatic activity, one histidine being protonated, the other unprotonated. In this case, NMR studies were possible because the histidine protons gave signals quite distinct from the mass of the other protons in the enzyme. Changes in the NMR signal as a function of pH implicated the two histidine residues in positions 12 and 119. This deduction was confirmed by inhibition studies of ribonuclease A using iodoacetate. Iodoacetate does not normally react chemically with histidine but it did react with two histidine residues in ribonuclease A, thereby simultaneously indicating that these two particular histidine residues were more reactive than normal. This was attributed to their being involved in hydrogen bonding within the active site that resulted in the weakening of their N-H bond, making it more reactive towards iodoacetate (Fig. 15.17).

15.4.6 Site-directed mutagenesis studies

Advances in molecular biology, and particularly in the ability to clone genes and express them in a particular vector, have opened up the possibility of producing variants of the enzyme in which a particular amino acid residue, thought to be involved in substrate binding and catalysis, is replaced by another amino acid. By studying the impact of the replacement of an ionisable or nucleophilic amino acid with an unreactive one on the catalytic properties of the enzyme, conclusions can be drawn about the role of the amino acid residue that has been replaced. Thus in the case of ribonuclease A, discussed above, replacement of either His12 or His119 has a deleterious effect on the catalytic properties of the enzyme. In principle it is also possible to produce variants that are more active than the native enzyme. Such studies are based on knowledge of the protein structure, function and mechanism of course and assume that the impact of the single amino acid replacement is confined to the active site and has not affected other aspects of the enzyme's structure. This needs to be confirmed by complementary structural studies, for example spectroscopic techniques. This rational redesign approach has resulted in the generation of a superoxide dismutase with an enhanced activity relative to the native enzyme and an isocitrate dehydrogenase with specificity different from that of the native form.

15.4.7 Catalytic mechanisms – outcomes of studies on ribonuclease A

The application of the various strategies outlined above to a wide range of enzymes has enabled mechanisms to be deduced for many of them. Crystallographic and site-directed mutagenesis studies have been particularly successful in providing detailed information about the stereochemical and electronic events involved in substrate binding and product formation. Such studies emphasise the importance of conformational changes that occur in the enzyme. Equally important is the role of hydrogen bonding between the substrate and key amino acid residues in the binding and catalytic sites in facilitating 'activation' of the substrate and the lowering of the reaction activation energy barrier. Many of the

mechanisms identified have other common features such as acid–base catalysis of the type well known in conventional organic chemistry. These findings are illustrated by studies on the enzyme ribonuclease A.

Ribonuclease A (RNase A)

RNase A is a small enzyme (molecular mass 13.68 kDa), consisting of only 124 amino acid residues including four histidine residues, which hydrolyses RNA, cleaving a ribose-phosphate ester bond attached to the ribose 5′ carbon, with a pyrimidine (cytosine or uracil) attached to the ribose in position 1′. The process has been shown to proceed via a 2′, 3′-phosphate cyclic diester that can be isolated and characterised. The evidence that allowed the mechanism of hydrolysis and the nature of the transition states to be deduced was obtained by application of a variety of strategies:

- pH-activity studies revealed a bell-shaped curve indicating the involvement of two histidine residues, one protonated and the other not, with pK_a values of 5.4 and 6.4, respectively.
- NMR studies gave signals for two protons from two histidine residues displaced from the remainder of the proton NMR signals (Section 15.4.5).
- Affinity labelling studies using iodoacetate indicated the involvement of His12 and His119. Similar studies using fluorodinitrobenzene indicated the involvement of Lys41 and possibly Lys7.
- Site-directed mutagenesis studies showed that Lys7, but not Lys41, could be replaced without loss of activity.
- Crystallographic studies revealed a cleft in the molecular structure into which the region of the RNA to be cleaved could bind. His12 and His119 were in close proximity but on opposite sides of the cleft. Lys7, Lys41 and Lys66 were in the same region as Asp121, which was adjacent to His119. It was deduced that the positively charged Lys residues would interact with the negatively charged phosphate groups of the RNA backbone and that Asp121, with its negative charge, would be involved in the transfer of a proton from water.
- Molecular modelling of the active site revealed that the pyrimidine group cytosine or uracil fits into the cleft in such a way as to form two hydrogen bonds with Thr45. Replacement of the pyrimidine by a purine (adenine or guanine) prohibited the formation of these bonds, thereby explaining the specificity for the pyrimidines.
- Model substrate studies showed that the enzyme cleaved a P-O ester bond attached to the ribose 5′-carbon and that the process proceeded via a 2′,3′-phosphate cyclic diester that could be isolated.

This evidence of the nature of the RNase A catalytic action enabled a probable catalytic mechanism to be deduced. It is summarised in Fig. 15.17. It is an example of acid–base catalysis that is common for hydrolase enzymes. Each of the two key histidine residues acts as both an acid and a base during the reaction sequence.

Confirmation of a pentavalent phosphoryl intermediate came from the fact that RNase A is competitively inhibited by uridine vanadate, in which the vanadium atom is pentavalent. The lack of a 2′-hydroxyl group in DNA (containing 2′-deoxyribose) prevents the formation of the pentavalent intermediates and thereby explains why RNase A does not hydrolyse DNA.

Multienzyme complexes

Studies on multienzyme complexes, including tryptophan synthase and carbamyl phosphate synthase, have demonstrated that the active site of one enzyme is coupled to that of the next enzyme in the metabolic sequence by means of allosteric conformational changes. The reaction products are channelled from one active site to the next by means of an intermolecular tunnel. In the case of tryptophan synthase, which is an $(\alpha\beta)_2$-complex in which the α- and β-subunits catalyse separate reactions, the tunnel is approximately 25 Å in length whereas that in carbamoyl phosphate synthase is approximately 100 Å long. (Note that angstroms are more commonly used for structural dimensions rather than the more correct nanometres.) The tunnels protect reactive intermediates from coming into contact with the external environment and reduce their transit time to the next active site. In the case of both enzymes the tunnels are formed prior to the binding of the initial substrates but with some other multienzyme complexes the tunnels are formed after the substrates bind to the active site.

15.5 CONTROL OF ENZYME ACTIVITY

15.5.1 Control of the activity of individual enzymes

The activity of an enzyme can be regulated in two ways:

- by alteration of the kinetic conditions under which existing enzyme is operating;
- by alteration of the amount of enzyme present either by promoting enzyme synthesis or enzyme degradation.

The latter option is inherently long-term and will be discussed later. In contrast, there are several mechanisms by which the activity of an enzyme can be altered almost instantaneously:

- *Product inhibition:* Here the enzyme product acts as an inhibitor of the reaction so that, unless the product is removed by further metabolism, the reaction will cease. An example is the inhibition of hexokinase by glucose 6-phosphate. Hexokinase exists in four isoenzyme forms I, II, III and IV. The first three isoforms all have a low K_m for glucose (about 10–100 μM) and are inhibited by glucose 6-phosphate, whereas isoform IV has a higher K_m (10 mM) and is not inhibited by glucose 6-phosphate. Isoform IV is confined to the liver, where its higher K_m allows it to deal with high glucose concentrations

following a carbohydrate-rich meal (see below). The other three isoforms are distributed widely and do not encounter such high glucose concentrations as those found in the liver. Thus their lower K_m values allow them to work optimally under their prevailing physiological conditions.

- *Allosteric regulation:* Here a small molecule that may be a substrate, product or key metabolic intermediate such as ATP or AMP alters the conformation of the catalytic site as a result of its binding to an allosteric site (Section 15.3.3). A good example is the regulation of 6-phosphofructokinase discussed earlier.
- *Reversible covalent modification:* This may involve adenylation of a tyrosine residue by ATP (e.g. glutamine synthase), the ADP-ribosylation of an arginine residue by NAD^+ (e.g. nitrogenase) but most frequently involves the phosphorylation of specific tyrosine, serine or threonine residues by a protein kinase. Most significantly, phosphorylation is reversible by the action of a phosphatase. Phosphorylation introduces the highly polar γ-phosphate group of ATP that is capable of inducing conformational changes in the enzyme structure such as to either activate or deactivate the enzyme.

Reversible covalent modification is quantitatively the most important of the three mechanisms. A very large number of protein kinases have been identified and shown to be of three basic types:

- *Receptor protein kinases:* These are located on the cytoplasmic side of a cell membrane receptor that is activated as a result of the binding to an extracellular site of a hormone, neurotransmitter or other signalling ligand released by a distant cell. Ligand activation of the receptor most commonly results in the dimerisation of the receptor and the autophosphorylation of the latent protein kinase site. This autophosphorylation of the receptor protein activates the protein kinase site and simultaneously creates a binding site for a number of intracellular enzymes that have a key cell-signalling role, resulting in their phosphorylation and a resulting modification of their activity. This is discussed in detail in Section 16.5.3.
- *Non-receptor protein kinases:* Members of this large group of kinases have vital roles in the control of cell differentiation, proliferation and death. The best studied are the Src family. They share a common structure and are all activated as a result of an initial dephosphorylation followed by phosphorylation by a receptor protein kinase of the type discussed above.
- *Second-messenger-coupled kinases:* Members of this important group of kinases are activated or deactivated by second messengers released by an intracellular membrane-bound enzyme that is activated by a G-protein- coupled receptor. The second messenger may be cAMP, cyclic GMP (cGMP), Ca^{2+} or diacylglycerol (for further details, see the following section and Chapter 16). Examples include cAMP-dependent protein kinase involved in glycogenolysis, protein kinase C (activated by Ca^{2+} and diacylglycerol) involved in the phosphorylation of the epidermal growth factor (EGF) receptor, and Ca^{2+}/calmodulin protein kinases involved in the regulation of other kinases such as phosphorylase kinase.

A characteristic feature of many of these various kinases is that they are involved in a cascade of enzyme reactions such as glycogenolysis and glycogenesis, which will be discussed in the following section. Such cascades offer the opportunity for fine metabolic control and a large amplification of the original signal received by the membrane receptor.

15.5.2 Control of metabolic pathways

A large proportion of the thousands of enzymes in a cell are involved in the promotion of coordinated chemical pathways such as glycolysis, the citric acid cycle and the biosynthesis of fatty acids and steroids. Enzymes linked in a coordinated pathway are frequently clustered in one of three ways, namely:

- by being located in the same compartment of the cell,
- by being physically associated as a multienzyme complex such as that of the fatty acid synthase of *E. coli*,
- by being membrane bound, such as the enzymes of the electron transport system.

This clustering facilitates the transport of the product of one enzyme to the next enzyme in the pathway.

Identification of rate-controlling enzymes

The individual enzymes in a metabolic pathway combine to produce a given flow of substrates and of products through the pathway. This flow is referred to as the metabolic flux. Its value is determined by factors such as the availability of starting substrate and cofactors but above all by the activity of the individual enzymes. Studies have revealed that the enzymes in a given pathway do not all possess the same activity. As a consequence one, or at most a small number, with the lowest activity determine the overall flux through the pathway. In order to identify these rate-controlling enzymes three types of study need to be carried out:

- *in vitro* kinetic studies of each individual enzyme conducted under experimental condition as near as possible to those found *in vivo* and such that the enzyme is saturated with substrate (i.e. such that $[S] > 10K_m$),
- studies to determine whether or not each individual enzyme stage operates at or near equilibrium *in vivo*,
- studies to determine the flux control coefficient for each enzyme.

A reaction that is not at or near equilibrium, and which is therefore associated with a large free energy change, is potentially a rate-limiting enzyme, since the most probable reason for the non-establishment of equilibrium is the lack of adequate enzyme activity. To test for a non-equilibrium reaction it is necessary to analyse the concentration of each substrate and product *in vivo*. This is normally done by stopping all further reactions by denaturing the enzymes by the addition of a suitable denaturant to the *in vivo* test system and then analysing the analytes by a technique such as chromatography or NMR.

Table 15.3 Rate-controlling tests on the enzymes of the glycolytic pathway

Enzyme	Activity	Equilibrium	Flux control coefficient *C*
Hexokinase	Low	Non-	High
Phosphoglucomutase	High	Near	Low
6-Phosphofructokinase	Low	Non-	High
Fructose bisphosphatase aldolase	High	Near	Low
Triosephosphate isomerase	High	Near	Low
Glyceraldehyde-3-phosphate dehydrogenase	High	Near	Low
Phosphogylcerate kinase	High	Near	Low
Phosphoglycerate mutase	High	Near	Low
Enolase	High	Near	Low
Pyruvate kinase	Low	Non-	Medium

For each enzyme in the pathway it is possible to calculate a flux control coefficient, *C*, which measures the impact of a change in the activity of that enzyme in the cell, under the prevailing physiological conditions, on the flux of reactants through the whole pathway. Values for *C* can vary between 0 and 1. A flux control coefficient of 1 means that the flux through the pathway varies in proportion to the increase in the activity of the enzyme whereas a flux control coefficient of zero means that the flux is not influenced by changes in the activity of that enzyme. The sum of all *C* values for a given pathway is 1 so that the higher a given *C* value the greater is the impact of that enzyme on the flux through the pathway. These *C* values are therefore highest for the rate-determining enzymes.

The application of these three tests to the enzymes in the glycolytic pathway is illustrated in Table 15.3. The actual quantitative values for the three test parameters vary between cell types in a given organism and between cells of a given type in different organisms. It can be seen from Table 15.3 that in the glycolytic pathway three of the 10 enzymes involved in the conversion of glucose to pyruvate have a potential rate-limiting activity and do not achieve equilibrium but are associated with a large negative free energy change and are therefore effectively irreversible. The same three enzymes have the largest *C* values. Studies have revealed that all three enzymes are subject to various control mechanisms and all contribute to the control of flux through the glycolytic pathway.

Hexokinase exists in four isoenzyme forms, the first three of which are subject to inhibition by glucose 6-phosphate, the product of the reaction. Isoenzyme IV (also known as glucokinase) is not subject to this type of inhibition and has a higher K_m for glucose than have the other three forms. It is confined mainly to the liver, where it is able to metabolise high concentrations of glucose, the resulting glucose 6-phosphate being diverted to glycogen biosynthesis via glucose 1-phosphate. In some tissues the limiting activity of hexokinase is bypassed by the provision of glucose 6-phosphate from glycogen via glucose 1-phosphate.

The activity of pyruvate kinase is regulated allosterically, being inhibited by ATP and activated by AMP and fructose 1,6-bisphosphate. In muscle, pyruvate

kinase is present in large amounts, hence minimising its rate-limiting constraint. The fact that pyruvate kinase is located at the end of the pathway makes it unlikely that it will have a major role in the regulatory control of glycolysis.

As discussed earlier (Section 15.3.3), 6-phosphofructokinase (PFK) is subject to allosteric control by a number of allosteric effectors that are related to the energy status of the cell. The principal activators are AMP and fructose 2,6-bisphosphate, whilst ATP is an activator at low concentrations but an inhibitor at higher concentrations (1 mM). AMP activates the enzyme by releasing it from the inhibitory control of ATP by binding to the R state, disturbing the equilibrium away from the T state that contains the ATP inhibitor site. The balance of control exerted by ATP and AMP is thus determined by their relative concentrations. This in turn is influenced by the enzyme adenylyl kinase, which catalyses the reaction:

$$2ADP \rightleftharpoons ATP + AMP$$

ATP is normally present in a cell at much higher concentrations than the other two nucleotides and, as a consequence, a small decrease in the concentration of ATP that is too small to relieve the inhibitory effect of ATP on PFK results in a proportionally much larger change in AMP concentration, which is normally only about 2% of that of ATP. This large percentage increase in the concentration of AMP allows it to exert a powerful activator effect on PFK, hence facilitating increased glycolytic flux.

Additional control of glycolytic flux by PFK is exerted by its involvement in a substrate cycle with the enzyme fructose bisphosphatase (FBP) that is part of the gluconeogenesis pathway from pyruvate to glucose (Fig. 15.18). Both reactions are strongly exergonic and essentially irreversible. Although AMP acts as a powerful activator of PFK, it acts as a potent inhibitor of FBP and hence plays a reciprocal role in the control of these opposing pathways. PFK converts D-fructose 6-phosphate to D-fructose 1,6-bisphosphate and simultaneously converts ATP to ADP, whilst FBP converts D-fructose 1,6-bisphosphate to D-fructose 6-phosphate and inorganic phosphate. The net result is apparently only the hydrolysis of ATP but in fact it results in a proportionally large increase in AMP concentration via adenylyl kinase. As discussed above, this produces a large increase in flux through the glycolytic pathway by the activation of PFK and inhibition of FBP. A two-fold increase in AMP concentration can increase the glycolytic flux by 200-fold. However, the regulatory importance of changes in AMP concentration is not confined to its stimulation of glycolytic flux. Equally important is the fact that decreases in AMP concentration result in the ATP inhibition of PFK activity becoming dominant, resulting in the virtual switching off of the glycolytic pathway and a concomitant increase in glycogen biosynthesis.

Although AMP is a significant regulator of the activity of PFK it is not the most potent activator of the enzyme. That role, at least in liver, is held by D-fructose 2,6-bisphosphate (F26BP) which is not an intermediate of glycolysis, although it is isomeric with fructose 1,6-bisphosphate, the product of PFK. F26BP activates PFK at concentrations as low as 1 μM. F26BP is part of another substrate cycle involving the enzymes phosphofructokinase-2 (PFK-2), which converts D-fructose

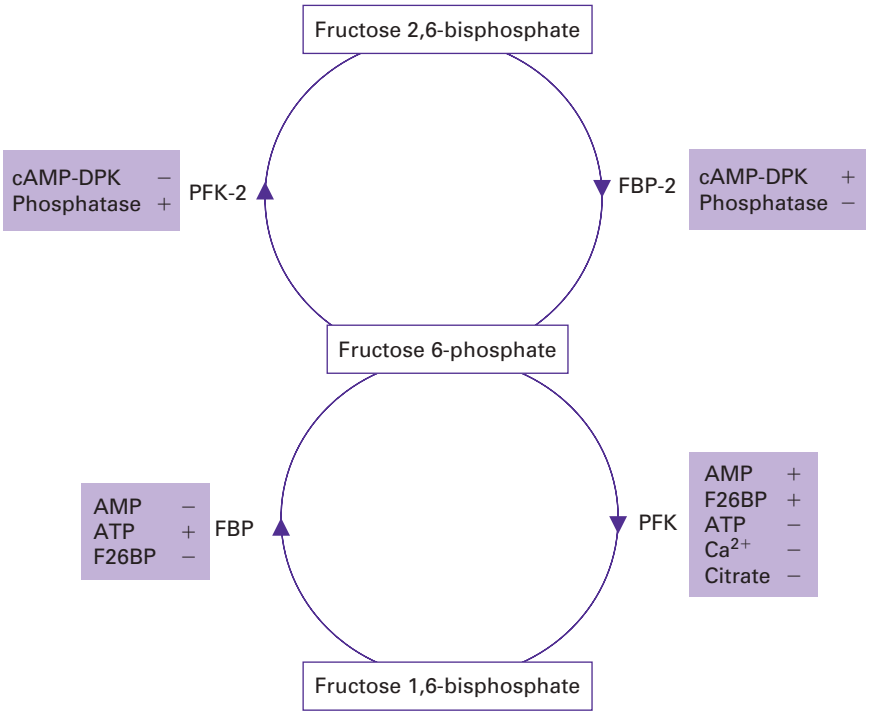


Fig. 15.18. Regulation of phosphofructokinase (PFK). Two substrate cycles each centred on fructose 6-phosphate are involved. Different enzymes promote the forward and reverse reactions of each cycle so that all reactions are exergonic (negative free energy changes). Each enzyme is subject to activation (+) or inhibition (−) either by allosteric effectors or by phosphorylation/dephosphorylation. The importance of each regulatory mechanism varies between organisms and between different tissues in a given organism. cAMP-DPK, cyclic-AMP-dependent protein kinase; F26BP, fructose 2,6-bisphosphate.

6-phosphate to D-fructose 2,6-bisphosphate, and fructose bisphosphatase-2 (FBP-2) that converts the bisphosphate back to the 6-phosphate. Interestingly, the two enzyme activities are located in different domains of the same dimeric protein. Both are subject to regulation by phosphorylation/dephosphorylation. FBP-2 is activated by phosphorylation and PFK-2 by dephosphorylation. Under cellular conditions in which the glucose concentration is high, PFK-2 is activated by dephosphorylation by an activated phosphatase thus increasing the concentration of F26BP, which in turn activates PFK and increases glycolytic flux. At the same time, FBP-2 is deactivated by dephosphorylation by the same phosphatase. When the glucose level is low, the cycle operates in reverse. The operation of this substrate cycle is the main mechanism that ensures that glycolysis and gluconeogenesis do not proceed *in vivo* at the same time.

Isoforms of PFK-2 and FBP-2 have been identified in different tissues. They differ in their affinity (K_s) for their substrates and in their sensitivity to regulation by phosphorylation/dephosphorylation. This rationalises the observation that the

fine mechanistic detail for the control of PFK activity and hence of the regulation of glycolysis varies between different mammalian tissues.

In general, substrate cycles are an important means by which the activity of metabolic pathways is controlled. They operate at the expense of energy (ATP) and may simultaneously determine the relative importance of branch points in bidirectional pathways.

15.5.3 Signal amplification

The substrate cycles discussed above enable opposing pathways to be controlled and small changes in the concentration of ATP to be amplified in terms of concomitant changes in AMP, which is a key allosteric regulator of rate-limiting enzymes. This concept of amplification is important in the fine control of metabolic pathways and in the response of cells to hormone and neurotransmitter signals. Amplification is commonly achieved by a series of stages in which linked enzymes are themselves the substrate of a reaction, commonly based on phosphorylation or dephosphorylation, as a result of which the enzymes are either activated or deactivated. Such a series of reactions is referred to as a metabolic cascade and its merit is that it affords the opportunity for a large amplification of an original biochemical signal. The mobilisation of glycogen as glucose 1-phosphate by phosphorylase provides a good illustration of this principle. The components of this phosphorylase cascade are a membrane receptor that receives the original signal in the form of a hormone, neurotransmitter or similar, a G_s protein, adenylyl cyclase (also known as adenylate cyclase), cAMP-dependent protein kinase, phosphorylase kinase, phosphorylase and glycogen (Fig. 15.19). cAMP released from adenylyl cyclase, as a result of its activation by a G_s protein (see Section 16.5.2), activates cAMP-dependent protein kinase, which in its inactive form is a tetramer consisting of two regulatory (R) and two catalytic (C) subunits. Two cAMP molecules bind to each of the R subunits in a positively cooperative manner, causing them to dissociate:

$$\underset{\text{inactive form}}{R_2C_2} + 4\text{cAMP} \longrightarrow \underset{\text{active form}}{2\text{R-2cAMP}} + 2\text{C}$$

The intracellular concentration of cAMP determines the proportion of cAMP-dependent protein kinase that is present in the active form. It is this form of the kinase that, in the presence of ATP, phosphorylates and thereby activates phosphorylase kinase:

$$\underset{\text{inactive}}{\text{phosphorylase kinase}} + \text{ATP} \xrightarrow{\text{C unit}} \underset{\text{active}}{\text{phosphorylated phosphorylase kinase}} + \text{ADP}$$

Phosphorylase kinase is a tetrameric protein with four different subunits, α, β, γ and δ. The γ-subunit contains the catalytic kinase site, the other three subunits having a regulatory role. The δ-subunit is calmodulin, a Ca^{2+}-binding protein

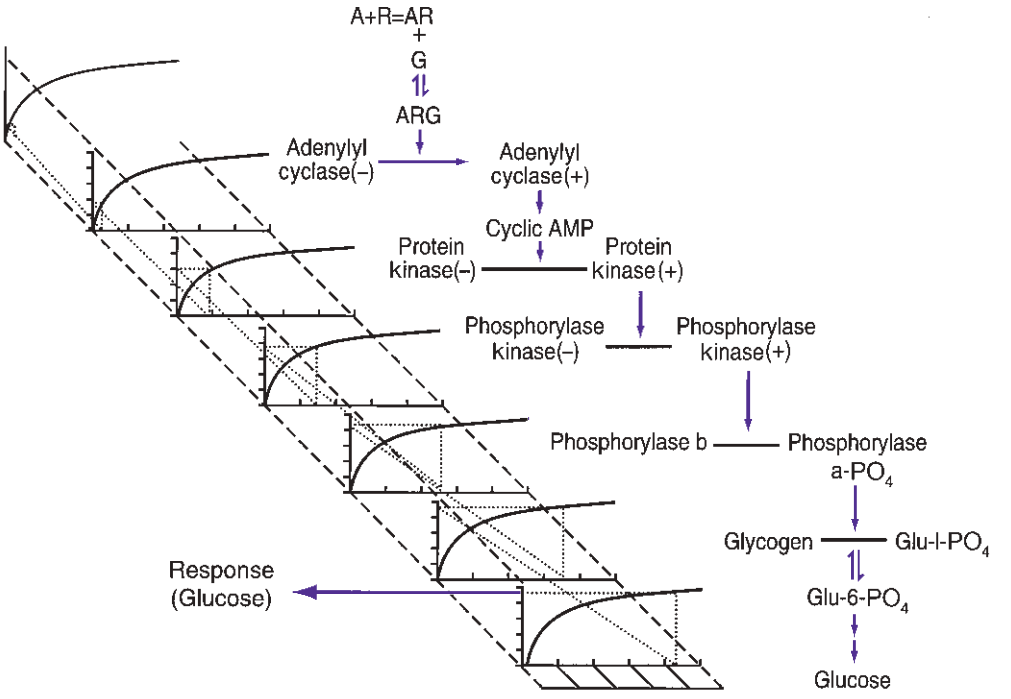


Fig. 15.19. A typical biochemical cascade showing the stages from the initial binding of the first messenger (A) to its cell-surface receptor, the resultant activation of a G-protein (G) by the AR complex, through the activation of adenylyl cyclase and the release of cAMP to the final cellular response, namely the release of glucose. Each step in the cascade is represented by a hyperbolic function. The initial low receptor occupancy triggers a sequence of progressively amplified responses so that the final release of glucose is nearly a maximal response. (Reproduced from T. Kenakin (1997), *Molecular Pharmacology: A Short Course*, by permission of Blackwell Science Limited.)

that contains two Ca^{2+}-binding sites. Phosphorylase kinase is activated by the phosphorylation of the α- and β-subunits and by the binding of two Ca^{2+} to the δ-subunit. The binding of Ca^{2+} to the δ-subunit promotes the autophosphorylation of the enzyme at a site different from that phosphorylated by cAMP-dependent kinase. The activated phosphorylase kinase activates phosphorylase b (a dimer) by the phosphorylation of Ser14 on each subunit, causing conformational changes and dimerisation to a tetramer to give phosphorylase a, which degrades glycogen to glucose 1-phosphate. Most interestingly, phosphorylase b can also be activated allosterically by AMP, two molecules of which are capable of inducing conformation changes to give phosphorylase a but by an induction mechanism different from that brought about by phosphorylation. ATP and glucose 6-phosphate can induce the reverse allosteric change, deactivating the enzyme.

At each step in the phosphorylase cascade there is amplification of at least 100-fold, moving the associated dose–response curve nearer the maximum (Fig. 15.19). Thus occupation of only a very small percentage of the membrane receptors is needed to produce a final metabolic response approaching the maximum. It is

evident that the larger the number of components in the cascade, the greater is the potential for amplification.

The mobilisation of glycogen is reversed by glycogen synthase, which is inactivated by phosphorylation by phosphorylase kinase and activated by phosphoprotein phosphatase-1, which simultaneously inactivates phosphorylase kinase and glycogen phosphorylase a. Phosphoprotein phosphatase-1 is itself subject to control by phosphorylation/dephosphorylation.

15.5.4 Long-term control of enzyme activity

The forms of control of enzyme activity discussed so far are essentially short- to medium-term control in that they are exerted in a matter of seconds or a few minutes at the most. However, control can also be exerted on a longer time scale. Long-term control, exerted in hours, operates at the level of enzyme synthesis and degradation. Whereas many enzymes are synthesised at a virtually constant rate and are said to be constitutive enzymes, the synthesis of others is variable and is subject to the operation of control mechanisms at the level of gene transcription and translation. One of the best-studied examples is the induction of β-galactosidase and galactoside permease by lactose in *E. coli*. The expression of the *lac* operon is subject to control by a repressor protein produced by the repressor gene (the normal state) and an inducer, the presence of which causes the repressor to dissociate from the operator, allowing the transcription and subsequent translation of the *lac* genes. The lac repressor protein binds to the lac operator with a K_i of 10^{-13} M and a binding rate constant of $10^7\,(\mathrm{M})^{-1}\,\mathrm{s}^{-1}$. This rate constant is greater than that theoretically possible for a diffusion-controlled process and indicates that the process is facilitated in some way, possibly by DNA.

The metabolic degradation of enzymes is the same as that of other cellular proteins, including membrane receptors. It is a first-order process characterised by a half-life. The half-life of enzymes varies from a few hours to many days. Interestingly, enzymes that exert control over pathways have relatively short half-lives. The precise amino acid sequence of a protein is thought to influence its susceptibility to proteolytic degradation. N-terminal Leu, Phe, Asp, Lys and Arg, for example, appear to predispose the protein to rapid degradation. Proteins for proteolytic degradation are initially 'tagged' by a small protein (76 amino acid residues), called ubiquitin (Ub), which requires ATP and is able to form an enzyme-catalysed peptide-like bond with the C-terminal end of the protein to be degraded. Ubiquitin may either mono-ubiquitinate or poly-ubiquitinate a protein and the functional consequences vary. Mono-ubiquitination leads to the 'trafficking' of the protein, a process that is fundamental to the cycling of receptors (Section 16.6.2), whereas poly-ubiquitination leads to degradation. The interaction between ubiquitin and a protein involves a series of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), ubiquitin protein ligases (E3s) and deubiquitylating enzymes (DUBs). The ubiquitin protein ligases play a key role in the whole process as they recognise the target protein. Results from the Human Genome Project provide evidence for the existence of several hundred of these E3 enzymes.

The poly-ubiquitinated protein is degraded by a multicatalytic complex based on a 20 S proteasome (the S stands for Svedberg, see Section 3.5.3). Proteasomes are multisubunit proteases with a cylindrical core that has a 'lid' at both ends. The catalytic sites are within the core cylinder. The 20 S proteasome consists of 14 α-subunits and 14 β-subunits arranged in four rings, each of seven units. The proteolytic activity is located in the β-subunits at five sites that lie in the core. Entry of the substrate protein into the cylindrical core is controlled by a number of activators and either may proceed sequentially, starting from one end of the protein, or may involve a 'hairpin' conformation of the protein entering the proteasome, allowing limited proteolysis of an internal segment. Proteolysis is ATP dependent and involves an additional 19 S proteasome that contains ATPase sites. This 19 S proteasome combines with the 20 S proteasome to form a 26 S proteasome that promotes the cleavage of the peptide bonds, with the concomitant hydrolysis of ATP. In addition to the proteasome route for enzyme degradation there is also a lysosomal route that does not require pre-ubiquitination of the enzyme.

The balance between enzyme *de novo* synthesis and proteolytic degradation coupled with the regulation of enzyme activity enables the amount and activity of enzymes present in a cell to be regulated to meet fluctuating cell and whole organism needs. There is growing evidence to indicate that ubiquitination/deubiquitination is as important as phosphorylation/dephosphorylation for cellular homeostasis.

15.6 SUGGESTIONS FOR FURTHER READING

General texts

BUGG, T. (2004). *An Introduction to Enzyme and Coenzyme Chemistry*, 2nd edn. Blackwell Science, Oxford. (A readable text that emphasises the chemical principles underlying enzymology.)

FELL, D. (1997). *Understanding the Control of Metabolism*. Portland Press, London. (An authoritative coverage of this important topic in which the underlying mathematical concepts are carefully explained.)

Review articles

CHEN, R. (2001). Enzyme engineering: rational redesign versus directed evolution. *Trends in Biotechnology*, **19**, 13–14.

CIECHANOVER, A. and BEN-SAADON, R. (2004). N-terminal ubiquitination: more protein substrates join in. *Trends in Cell Biology*, **14**, 103–106.

DANIEL, R. M., DANSON, M. J. and EISENTHAL, R. (2001). The temperature optima of enzymes: a new perspective on an old phenomenon. *Trends in Biochemical Sciences*, **26**, 223–225.

FÖSTER, A. and HILL, C. P. (2003). Proteasome degradation: enter the substrate. *Trends in Cell Biology*, **13**, 550–553.

HUANG, H., HOLDEN, H. M. and RAUSHEL, F. M. (2001). Channeling of substrates and intermediates in enzyme-catalysed reactions. *Annual Reviews of Biochemistry*, **70**, 149–180.

Chapter 16

Cell membrane receptors

16.1 RECEPTORS FOR CELL SIGNALLING

Cells within multicellular organisms need to be able to communicate with each other in order to coordinate essential functions such as growth and differentiation and to respond to changes in their external environment. Cells in physical contact with each other can interact by the exchange of small molecules via gap junctions but cells physically distant from each other, with the extreme examples being found in plants and animals, need an effective communication system. This is achieved by the release of ligand signalling molecules by the signalling cells and the specific recognition of these ligands by protein receptors either embedded within and spanning the cell membrane or located in the cytoplasm of the target cells. Most commonly the ligand is water soluble and therefore incapable of diffusing across the cell membrane. It therefore binds to a ligand-binding site exposed on the extracellular side of the receptor. The binding initiates a sequence of events, in many cases involving protein–protein interactions at the membrane interface, which result in the cellular response. Examples of such ligands include amines, amino acids, peptides and proteins. However, some ligands are lipid soluble and can diffuse freely across the membrane and bind to cytosolic receptors. The receptor–ligand complexes subsequently diffuse across the nuclear membrane and accumulate in the nucleus where they modulate DNA transcription. For this reason, the receptors are referred to as nuclear receptors. Examples of ligands acting in this way are steroid hormones (progesterone, oestrogen, testosterone) and non-steroid hormones (thyroxine and triiodothyronine).

A ligand acting on a cell membrane receptor is often referred to as a first messenger, since with many membrane receptors ligand binding does not directly result in the desired intracellular response. Rather, it results in the activation of the intracellular region of the receptor that then either:

- initiates a series of intracellular protein activation processes that climax in the cellular response, or
- interacts with a second membrane-bound protein that in turn promotes the generation or release from intracellular stores of one or more of a group of molecules including cAMP and Ca^{2+}, collectively called second messengers,

that diffuse to, and react with, various protein targets, modifying their activity and thereby stimulating the cellular response.

In both cases, a series of reactions, collectively referred to as a cascade, is involved in the transduction (linking) of the original signal received by the receptor to the eventual cellular response. One of the advantages of a cascade system is that it results in a large amplification of the cellular response relative to that which could possibly have been produced by the first messenger acting alone (for further details see Section 15.5.3). The signalling ligand has no other physiological function in addition to its role of promoting the formation of the active conformation of the receptor. With two of the three main classes of receptor, the receptor–ligand complex is internalised by endocytosis, causing the ligand to dissociate under the acidic conditions prevailing in the endocytic vesicles. The vacated receptor may be either recycled or degraded (Section 16.6.2).

16.2 QUANTITATIVE ASPECTS OF RECEPTOR–LIGAND BINDING

16.2.1 Dose–response curves

Pharmacological objectives

Much of the early work on the study of receptor–ligand binding was carried out on isolated tissue preparations and was aimed at the quantification and characterisation of the response as a function of the ligand concentration. The work was largely driven by the search for ligand blockers, which could mimic the binding of the physiological ligand but did not produce a cellular response. These ligand blockers had the potential for pharmacological exploitation. In the 1960s it was realised that if such blockers were sufficiently selective in their binding they might be able to distinguish between the various subclasses of the receptor that were believed to exist but which at the time had not been identified. The existence of subclasses of receptors would allow the development of blockers as therapeutic agents that would be tissue- and response-selective, thereby minimising undesirable side-effects. It was as a result of this approach that Sir James Black achieved his pioneering work on β-adrenergic and histamine H_2 receptors, which resulted respectively in the development of the blood pressure-lowering β-blocker propranolol and the gastric acid secretion H_2 receptor blocker cimetidine, used in the treatment of gastric and duodenal ulcers. As will be discussed in detail later, this approach to receptor classification, coupled with the newer technique of cloning and identification of encoding genes, has resulted in the identification of a plethora of receptor subtypes for most receptors.

Dose–response curves

The response of the membrane receptor to exposure to an increasing concentration (dose) of ligand is a curve that has three distinct regions:

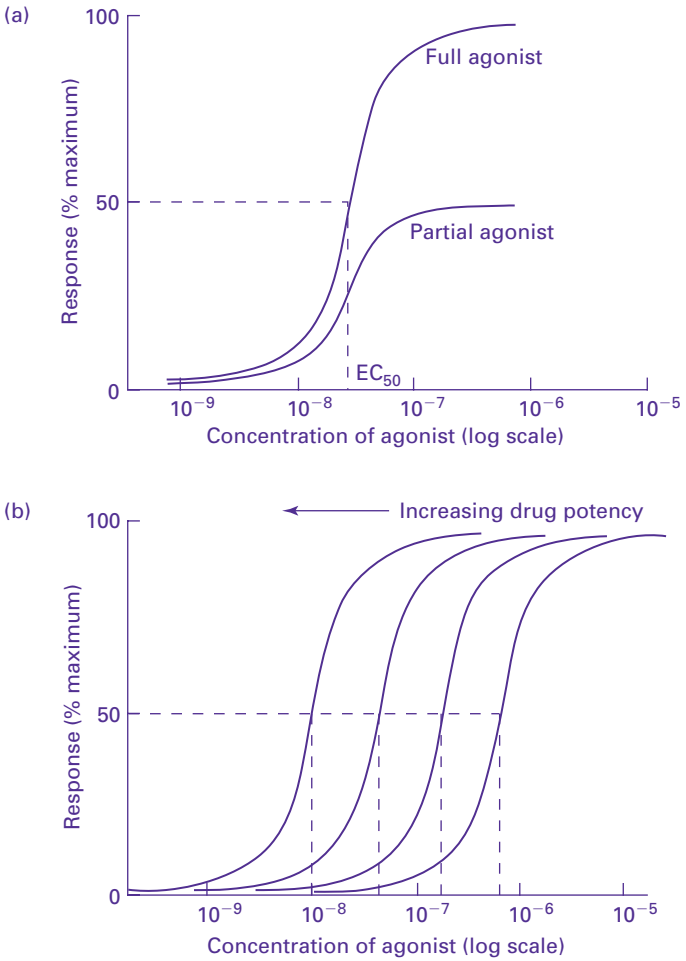


Fig. 16.1. Dose–response curves. (a) The biological effect (% maximum response) and the concentration of a full agonist are plotted on a logarithmic scale. An equipotent partial agonist has a lower efficacy than a full agonist – it cannot achieve the maximum response even when all available receptors are occupied. EC_{50} is the concentration of agonist that produces 50% maximum effect. (b) Dose–response curves for four full agonist drugs of different potencies but equal efficacy. (Reproduced from S. R. J. Maxwell and D. J. Webb (1999), Receptor functions. *Medicine*, **27**, 5–9, by permission of The Medicine Publishing Company.)

- an initial threshold below which little or no response is observed,
- a slope in which the response increases rapidly with increasing dose,
- a declining response with further increases in dose and a final maximum response.

Since such plots commonly span several hundred-fold variations in ligand concentration, they are best expressed in semilogarithmic form (Fig. 16.1).

Dose–response studies for a given receptor using ligands with a wide range of molecular structures have shown that receptors are capable of binding ligands

other than the physiologically active ligand and that the response to such binding is variable. As a result of such studies, receptor ligands have been categorised as follows:

- *Full agonists:* These ligands produce the same maximal response but differ in the dose required to achieve it (Figs. 16.1 and 16.2).
- *Partial agonists:* These ligands produce only a partial response even when present in large excess such that all the receptors are occupied (Figs. 16.1 and 16.2).
- *Antagonists:* These ligands produce no response. Three subclasses have been identified:
  - (i) *Competitive reversible antagonists:* The antagonist competes with the agonist for the same binding site so that the effect of the antagonist can be overcome by increasing the concentration of agonist (Fig. 16.2).
  - (ii) *Non-competitive reversible antagonists:* The antagonist binds at a different site on the receptor from that of the agonist so that the effect of the antagonist cannot be overcome by increasing the concentration of agonist.
  - (iii) *Irreversible competitive antagonists:* The antagonist competes with the agonist for the same binding site but the antagonist forms a covalent bond with the binding site so that its effect cannot be overcome by increasing the concentration of agonist.

As a result of this classification of ligands, they can also be characterised by a number of parameters:

- *Intrinsic activity:* This is a measure of the ability of an agonist to induce a response by the receptor. It is defined as the maximum response to the test agonist relative to the maximum response to a full agonist acting on the same receptors. All full agonists, by definition, have an intrinsic activity of 1 whereas partial agonists have an intrinsic activity of <1.
- *Efficacy (e):* This is a measure of the inherent ability of an agonist to initiate a physiological response following binding to the receptor. The initiation of a response is linked to the ability of the agonist to promote the active conformational of the receptor. While all full agonists must have a high efficacy their efficacy values will not necessarily be equal, in fact values of *e* have no theoretical maximum value. Partial agonists have a low efficacy and antagonists have zero efficacy.
- *Potency:* This is a measure of the concentration of agonist required to produce the maximum effect: the more potent the agonist, the smaller the concentration required. The potency of an agonist is related to the position of the sigmoidal curve on the log dose axis. It is expressed in a variety of forms including the effective dose or concentration for 50% maximal response, ED_{50} or EC_{50}. On a semilogarithmic plot, the value emerges as pED_{50} or pEC_{50} value (i.e. $-\log_{10} ED_{50}$). Thus an agonist with EC_{50} of 3×10^{-5} M would have a pEC_{50} of 4.8.
- *Affinity:* This is a measure of the concentration of ligand required to produce 50% binding. This is the only parameter that can be used to characterise

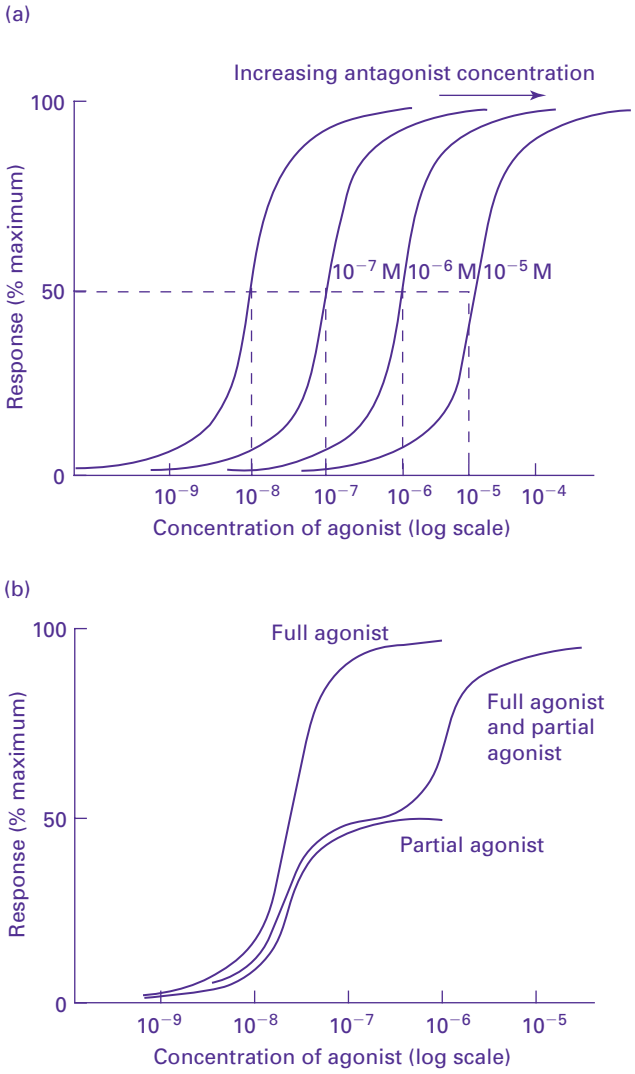


Fig.16.2. Receptor–ligand interactions. (a) In the presence of an antagonist, the dose–response curve of the full agonist is shifted to the right because the full agonist competes for receptor binding. The higher the concentration of antagonist, the greater is the shift. (b) In the presence of a partial agonist, the dose–response curve of the full agonist is shifted to the right. (Reproduced from S. R. J. Maxwell and D. J. Webb (1999), Receptor functions. *Medicine*, **27**, 5–9 by permission of The Medicine Publishing Company.)

antagonists. As will be shown in the following section, affinity is a reflection of both the rate of association of the ligand with the receptor and the rate of dissociation of the resulting complex. The rate of association is a reflection of the three-dimensional interaction between the two and the rate of dissociation a reflection of the strength of binding within the complex. Affinity can be

expressed by an affinity or binding constant, K_a, but is more commonly expressed as a dissociation constant, K_d, of the receptor–ligand complex, where K_d is equal to the reciprocal of K_a.

- *Selectivity:* This is a measure of the ability of an agonist to discriminate between receptor subtypes. This is particularly important from a therapeutic perspective.

Mechanisms of receptor activity

The existence of agonists and antagonists was originally explained by the two-state theory of receptors, also known as the del Castillo–Katz model. The model assumes that the occupied receptors can exist in two forms, one inactive (AR) and the other active (AR*) such that the two forms exist in equilibrium:

A	+	R	⇌	AR	⇌	AR*
agonist		vacant inactive receptor		occupied inactive receptor		occupied active receptor

It was envisaged that full agonists could readily induce conformational changes in R thereby creating the active R* state and displacing the equilibrium totally in favour of AR*, but partial agonists were less able to induce the change and hence could only partially displace the equilibrium. Antagonists could not induce the change at all. However, the discovery that some receptors are active even in the absence of agonists, and are said to possess constitutive activity, has led to the model being superseded by the conformational selection model. This model envisages that in the resting state (i.e. in the absence of any agonist) receptors exist as an equilibrium mixture of active (R*) and inactive (R) forms that can readily interconvert. The equilibrium may be predominantly in favour of the inactive state or such that a significant amount of the active state exists, thereby explaining the observed constitutive activity. According to this model, agonists preferentially bind to the R* state, thereby stabilising it and causing a concomitant displacement of the equilibrium, resulting in a greater proportion of the receptors being in the R* conformation. Similarly, partial agonists are capable of binding to both R and R* states, with variable preference for R* again resulting in an increase in the proportion of this conformation but smaller than that produced by full agonists. Support for this model has come from the identification of ligands that reduce the activity of receptors with constitutive activity. Since antagonists are believed to be unable to combine with the active state and do not produce any direct change in receptor activity, these ligands have been termed inverse agonists, since they have opposite or negative activity to that of agonists. They preferentially bind to the R state, stabilising it and displacing the equilibrium in its favour. Partial inverse agonists can bind to both states with a preference for the R state, thus decreasing the constitutive activity. Ligands with equal affinity for both states will not displace the equilibrium and are termed neutral antagonists. Most previously classified antagonists on re-evaluation against receptors possessing constitutive activity have been found to be inverse agonists, but of course it is

only possible to undertake such a re-evaluation with receptors displaying constitutive activity and at present these remain a minority. However, the technique of plasmon-waveguide resonance spectroscopy (Section 16.3.2) has recently proved to be an effective technique for distinguishing between these various classes of ligand.

The conformational selection model assumes that the various forms of the receptor (R, R*, AR, AR*) are in equilibrium so their relative proportions will be determined by the values of the associated equilibrium constants:

$$\begin{array}{lcccl} & & & \overset{K_1}{\rightleftharpoons} & \\ \text{(inactive)} & \text{R} & & & \text{R}^* \text{ (active)} \\ & + & & & + \\ & \text{L} & & & \text{L} \\ K_2 & \Vert & & & \Vert \quad K_3 \\ \text{(inactive)} & \text{LR} & & \underset{K_4}{\rightleftharpoons} & \text{LR}^* \text{ (active)} \end{array}$$

The important feature of this model is that the role of the ligand (L) is to bind to a particular receptor conformation, thereby stabilising it and causing a displacement of the equilibrium between the two states of the receptor. It is evident that the two-state model is a limiting case of this model and arises when the values of K_1 and K_3 are very small. The model does not require the agonist to induce the active conformation of the receptor. In this respect, receptors displaying this type of behaviour are reflecting the symmetry model of allostery put forward by Monod, Wyman and Changeux rather than the induced fit model proposed by Koshland (Section 15.5, Fig. 15.11). Receptors with constitutive activity have been identified among ligand-gated ion-channel and G-protein-coupled receptors.

16.2.2 Receptor–ligand binding parameters

The general process of the binding of a ligand to a receptor is initiated by the diffusion of the ligand to the surface of the receptor protein. Here the ligand locates the binding site by a series of random collisions within the two dimensions of the receptor surface in the membrane. The initial binding with the site may involve amino acid residues that are not functionally part of the final binding site, which most commonly consists of a few specific amino acid residues. This final binding can be represented as follows:

$$\begin{array}{ccccc} \text{R} & + & \text{L} & \overset{k_{+1}}{\underset{k_{-1}}{\rightleftharpoons}} & \text{RL} \\ \text{receptor} & & \text{ligand} & & \text{receptor–ligand complex} \end{array} \qquad (16.1)$$

where k_{+1} is the association rate constant and k_{-1} is the dissociation rate constant.

Saturation studies

If under the conditions of the binding studies the total concentration of ligand is very much greater than that of receptor (so-called saturation conditions), changes in ligand concentration due to receptor binding can be ignored but changes in the free (unbound) receptor concentration cannot. Hence if:

- $[R_t]$ is the total concentration of receptor that determines the maximum binding capacity
- $[L]$ is the free ligand concentration
- $[RL]$ is the concentration of receptor–ligand complex

then $[R_t]-[RL]$ is the concentration of free receptor.

At equilibrium, the forward and reverse reactions for ligand binding and dissociation (equation 16.1) will be equal:

$$k_{+1}([R_t]-[RL])[L] = k_{-1}[RL]$$

therefore

$$\frac{k_{-1}}{k_{+1}} = K_d = \frac{1}{K_a} = \frac{([R_t]-[RL])[L]}{[RL]} \tag{16.2}$$

where K_d is the dissociation constant for RL and K_a is the association or affinity constant. Rearranging gives:

$$[RL] = \frac{[L][R_t]}{K_d + [L]} \tag{16.3}$$

Determination of K_d

Equation 16.3 is of the form of a rectangular hyperbola that predicts that ligand binding will reach a limiting value as the ligand concentration is increased and therefore that receptor binding is a saturable process. The equation is of precisely the same form as equations 15.1 and 15.2, which define the binding of the substrate to its enzyme in terms of K_m and V_{max}. For the experimental determination of K_d, equation 16.3 is best converted to a linear form. Rearrangement of equation 16.3 gives the Scatchard equation (16.4):

$$\frac{[RL]}{[L]} = \frac{[R_t]}{K_d} - \frac{[RL]}{K_d} \tag{16.4a}$$

This equation predicts that a plot of $[RL]/[L]$ against $[RL]$ will be a straight line of slope $-1/K_d$, allowing K_d to be calculated. However, in many studies the relative molecular mass of the receptor protein is unknown so that the concentration term $[RL]$ cannot be calculated in molar terms. In such cases it is acceptable to express the extent of ligand binding in any convenient unit (B), e.g. pmoles $(10^6 \text{ cells})^{-1}$, pmoles $(\text{mg protein})^{-1}$ or more simply as a change in fluorescence (ΔF) under the defined experimental conditions. Since maximum binding (B_{max}) will occur when all the receptor sites are occupied, i.e. $[R_t] = B_{max}$, equation 16.4a can be written in the form:

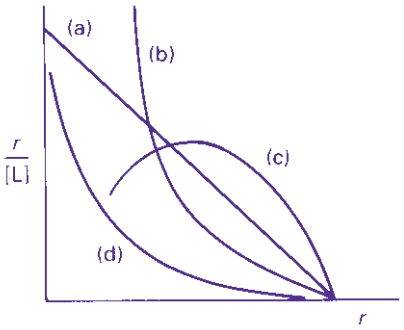


Fig. 16.3. Scatchard plot for (a) a single set of sites with no cooperativity, (b) two sets of sites with no cooperativity, (c) a single set of sites with positive cooperativity, and (d) a single set of sites with negative cooperativity.

$$\frac{B}{[L]} = \frac{B_{max}}{K_d} - \frac{B}{K_d} \tag{16.4b}$$

Hence a plot of $B/[L]$ against B will be a straight line, slope $-1/K_d$ and intercept on the y-axis of B_{max}/K_d (Fig. 16.3). In cases where the relative molecular mass of the receptor protein is known the Scatchard equation can be expressed in the form:

$$\frac{B}{[L]B_{max}} = \frac{n}{K_d} - \frac{B}{B_{max}K_d} \tag{16.5}$$

where n is the number of independent ligand-binding sites on the receptor.

The expression B/B_{max} is the number of moles of ligand bound to one mole of receptor. If this expression is defined as r, then:

$$\frac{r}{[L]} = \frac{n}{K_d} - \frac{r}{K_d} \tag{16.6}$$

In this case, a plot of $r/[L]$ against r will again be linear with a slope of $-1/K_d$ but in this case the intercept on the x-axis will be equal to the number of ligand-binding sites, n, on the receptor.

Alternative linear plots to the Scatchard plot are:

Lineweaver–Burk plot

$$\frac{1}{B} = \frac{1}{B_{max}} + \frac{K_d}{B_{max}} \times \frac{1}{[L]} \tag{16.7}$$

Hanes plot

$$\frac{[L]}{B} = \frac{K_d}{B_{max}} + \frac{[L]}{B_{max}} \tag{16.8}$$

In practice, Scatchard plots are most commonly carried out, although statistically they are prone to error, since the experimental variable B occurs in both the x and y terms so that linear regression of these plots overestimates both K_d and B_{max}. It can be seen from equation 16.2 that, when the receptor sites are half saturated,

i.e. $B = B_{max}/2$, then [L] = K_d. This is analogous to S = K_m when $v_o = V_{max}/2$ (Section 15.3.1). Hence K_d will have units of molarity.

The derivation of equation 16.2 is based on the assumption that there is a single set of homogeneous receptors and that there is no cooperativity between them in the binding of the ligand molecules. In practice, two other possibilities arise: first, that there are two distinct populations of receptors each with different binding constants; and, secondly, that there is cooperativity in binding within a single population. In both cases the Scatchard plot will be curvilinear (Fig. 16.3). If cooperativity is suspected, it should be confirmed by a Hill plot, which, in its non-kinetic form, is:

$$\log\left(\frac{Y}{1-Y}\right) = h \log [L] - \log K_d \qquad (16.9)$$

or

$$\log\left(\frac{B}{B_{max}-B}\right) = h \log [L] - \log K_d$$

where Y is the fractional saturation of the binding sites (from 0 to 1) and h is the Hill constant.

For a receptor with multiple binding sites that function independently, $h = 1$, whereas, for a receptor with multiple sites that are interdependent, h is either >1 (positive cooperativity) or <1 (negative cooperativity). Scatchard plots that are biphasic due to ligand multivalence (i.e. multiple binding sites), rather than receptor cooperativity, are sometimes taken to indicate that the two extreme, and approximately linear, sections of the curvilinear plots represent high affinity (high bound:free ratio at low bound values) and low affinity (low bound:free ratio at high bound values) sites and that tangents drawn to these two sections of the curve can be used to calculate the associated K_d and B_{max} values. This is incorrect, and the correct values can only be obtained from the binding data by means of careful mathematical analysis, generally undertaken by the use of special computer programs, many of which are commercially available.

Competitive binding experiments

An alternative approach to that described above for the determination of K_d values for an experimental ligand is to use the ligand as a competitive inhibitor of a second ligand, normally the physiological agonist, whose K_d value is known. This will give rise to a K_i value for the experimental ligand but this will be numerically equal to its K_d value. The most common approach is to use the physiological agonist in radiolabelled form and to use a range of concentrations of the experimental ligand with a fixed concentration of the radiolabelled ligand. A plot is then made of the extent of binding of the radiolabelled ligand against the log of the experimental ligand concentration. An IC_{50} value (the concentration required to inhibit the binding of the physiological ligand by 50%) is then calculated for the experimental ligand. From knowledge of the IC_{50} value the K_i value is calculated using the Cheng–Prusoff equation:

$$K_i = \frac{IC_{50}}{\{1 + ([L]/K_d)\}} \tag{16.10}$$

Determination of rate constants

To determine the dissociation rate constant, k_{-1} (units: time^{-1}) some of the receptor–ligand complex (B_0) is allowed to form, usually using radiolabelled ligand. The availability of the unoccupied receptors to the ligand is then blocked by the addition of at least 100-fold excess of unlabelled agonist or competitive antagonist and the decrease in the extent of binding (B_t) monitored as a function of time. The rate of release of the radiolabelled ligand from its binding site is given by the expression:

$$\frac{dB_0}{dt} = -k_{-1}B_0$$

and the equation governing the release by the expression:

$$B_t = B_0 e^{-k_{-1}t}$$

hence:

$$\log B_t = \log B_0 - 2.303k_{-1}t \tag{16.11}$$

Thus a plot of log B_t against time will give a straight line with a slope of $-2.303k_{-1}$, allowing k_{-1} to be estimated.

The association rate constant, k_{+1} (units: (mol dm^{-3})$^{-1}$ time^{-1}) is best estimated by the approach to equilibrium method, by which the extent of ligand binding is monitored continuously until equilibrium is reached under conditions that are such that [L] $\gg$ [R_t]. (This gives pseudo first-order conditions rather than second order. Under these conditions, [R_t] decreases with time but [L] remains constant.) Ligand binding increases asymptotically such that:

$$\log\left(\frac{B_{eq}}{B_{eq} - B_t}\right) = 2.303(k_{+1}[L] + k_{-1})t \tag{16.12}$$

Thus a plot of log [$B_{eq}/(B_{eq} - B_t)$] against time will be linear, with a slope of $2.303(k_{+1}[L] + k_{-1})$, where B_{eq} and B_t are the ligand binding at equilibrium and time t respectively. From knowledge of k_{-1} (obtained by the method discussed above) and [L], the value of k_{+1} can be calculated from the slope.

16.3 TECHNIQUES FOR THE STUDY OF RECEPTOR–LIGAND BINDING

16.3.1 Experimental design

General experimental problems

The rate and extent of receptor–ligand binding is influenced by such physical experimental variables as temperature, pH and ionic concentration. In addition,

there are some physiological problems that need to be taken into account. For example, if intact cells are the source of the receptor, it is essential to minimise receptor trafficking influences (Section 16.6.2) as otherwise receptor numbers may change during the study. This problem can be avoided in a number of ways including:

- carrying out the study at lowered temperatures, usually about 5 °C, that minimise trafficking processes,
- including an inhibitor of trafficking, such as phenylarsine oxide, in the incubation mixture,
- using isolated receptors or membrane fragments rather than whole cells.

Historically, the most common method of monitoring the progress of receptor–ligand binding has been to use a radiolabelled ligand and to measure the radioactivity associated with the receptor fraction of the ligand. However, this technique does not easily allow the determination of rate constants and is uneconomical if a large range of ligands is being screened against orphan receptors, i.e. those whose natural agonist is unknown, for example from cloned gene lines. For such reasons, fluorimetric techniques have become increasingly popular and for similar reasons, miniaturised ligand-binding assays based on protein microarray technology have been developed (Sections 8.5 and 16.3.2).

Receptor preparations

Preparations of receptors for ligand-binding studies may either leave the membrane intact or involve the disruption of the membrane and the release of the receptor with or without membrane fragments, some of which could form vesicles with variable receptor orientation and control mechanisms. A further potential problem of membrane disruption is that the process may expose receptors previously not within the cell membrane, thereby resulting in an overestimation of the number of available receptors. Such receptors could have been undergoing endocytosis (Section 16.6.2) or synthesis and insertion into the membrane.

Membrane receptor proteins show no or very little ligand-binding properties in the absence of phospholipid, so that a purified receptor protein must be introduced into a phospholipid vesicle for binding study purposes. In practice, common receptor preparations include:

- tissue slices, usually 5–50 μm thick cut with a cryostat and adhered to a gelatin-coated glass slide,
- isolated cells, produced by either the mechanical or the enzymatic (collagenase or trypsin) disruption of the whole tissue,
- cultured cells (Chapter 2), taking care to ensure their lack of contamination,
- cell membrane preparations, obtained by the use of a variety of methods for cell disruption coupled with differential centrifugation,
- solubilised receptor preparations, obtained by the use of detergents as the membrane disruption agents and purified by affinity chromatography using a competitive antagonist as the immobilised ligand,

- recombinant receptors, obtained using cell lines transfected with cloned receptor genes, increasingly of human origin.

Tissue slices are best for the study of receptor distribution and number by autoradiography or fluorescence spectroscopy, but the study of the kinetics of ligand binding in these preparations is complicated by the existence of diffusion barriers.

Isolated cells as a source of receptors are widely used for a wide range of biochemical studies including receptor post-translational modification, membrane insertion and downregulation, and the study of receptor–response coupling. However, binding studies using intact cells have a number of potential problems:

- the cells may contain enzymes capable of metabolising the experimental ligand thereby reducing its effective concentration,
- receptor trafficking (Section 16.6.2) and other cellular processes may affect receptor numbers and hence ligand binding,
- more than one type of cell may be present but the use of receptor gene cloning and expression in a specific cell line effectively overcomes this problem.

Cell membrane preparations are an experimentally useful source of receptors, but they lack the cytoplasmic components that may influence the regulation of ligand binding.

Recombinant receptors are increasingly being used for ligand binding studies, but care has to be taken to ensure that they have the same functional characteristics as the native receptor. It is particularly important to ensure that post-translational processes have been carried out and that, for example, the receptor protein has been correctly glycosylated.

Kinetic studies aimed at the determination of individual rate constants are best carried out using either isolated cells or the patch clamp technique (Section 16.3.2), since they avoid errors due to the diffusion of the ligand to the receptor. Studies of the number of receptors in intact tissue are best achieved by labelling the receptors with a radiolabel, preferably using an irreversible competitive antagonist and applying the technique of quantitative autoradiography. An alternative approach is to label the receptors with a positron emitter (e.g. ^{11}C) and to apply the technique of positron emission tomography (PET).

Ligands

By far the most common technique for the study of ligand–receptor interaction is the use of a radiolabelled ligand with isotopes such as ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{135}I. Generally, a high specific activity (Section 14.1.6) ligand is used, as this minimises the problem of non-specific binding (see below). The most effective alternative to the use of radiolabelled ligands is the use of fluorescence spectroscopy. This is discussed in Section 16.3.2.

For the study of ligand–receptor interactions that occur on a submillisecond timescale, special approaches need to be taken to deliver the ligand to the receptor (see stopped-flow and quench-flow methods in Section 15.2.3). One such approach is the use of so-called caged compounds. These possess no inherent

ligand properties but, on laser flash photolysis with light of a specific wavelength, a protecting group masking a key functional group is instantaneously cleaved releasing the active ligand.

Experimental procedures

The general experimental approach for studying the kinetics of receptor–ligand binding is to incubate the receptor preparation with the ligand under defined conditions of temperature, pH and ionic concentration for a specific period of time that is sufficient to allow equilibrium to be attained. The importance of allowing the system to reach equilibrium cannot be overstated as equations 16.1–16.9 do not hold if equilibrium has not been attained. Using an appropriate analytical procedure, the bound and unbound forms of the ligand are then quantified. This quantification may necessitate the separation of the bound and unbound fractions. The study is then repeated for a series of ligand concentrations to cover 10–90% of maximum binding at a fixed receptor concentration. The binding data are then analysed using equations 16.4–16.9, often in the form of a computer program.

Historically, ligand binding studies have been carried out on a manual basis but recent advances in molecular biology, especially gene cloning, have opened up the need for fast, high throughput screening techniques for large numbers of orphan receptors of unknown function but of potential pharmacological value. Some of the approaches taken to solve this need are discussed later.

Non-specific binding

Irrespective of the analytical procedure chosen to separate the bound and free ligand, a general problem is the non-specific binding of the ligand to sites other than the specific ligand binding site(s). Such non-specific binding may involve the membrane lipids and other proteins either located in the membrane or released by the isolation procedure. The characteristic of non-specific binding is that it is non-saturable but is related approximately linearly to the total concentration of the ligand. Thus the observed ligand binding is the sum of the saturable (hyperbolic) specific binding to the receptor and the non-saturable (linear) binding to miscellaneous sites. The specific binding component is usually obtained indirectly by carrying out the binding studies in the presence of an excess of non-labelled ligand (agonist or antagonist) if a labelled ligand is being used to study binding. The presence of the excess unlabelled ligand will result in the specific binding sites not being available to the labelled ligand and hence its binding would be confined to non-specific sites (Fig. 16.4). In practice, a concentration of the competitive ligand of at least 1000 times its K_d must be used and confirmation that under the conditions of the experiment, non-specific binding was being studied would be sought by repeating the study using a range of different and structurally dissimilar competitive ligands that should give consistent estimates of the non-specific binding.

Separation of bound and free ligand

Binding studies based on the use of a radiolabelled ligand necessitate the separation of the receptor-bound and free ligand fractions. This can be achieved in a

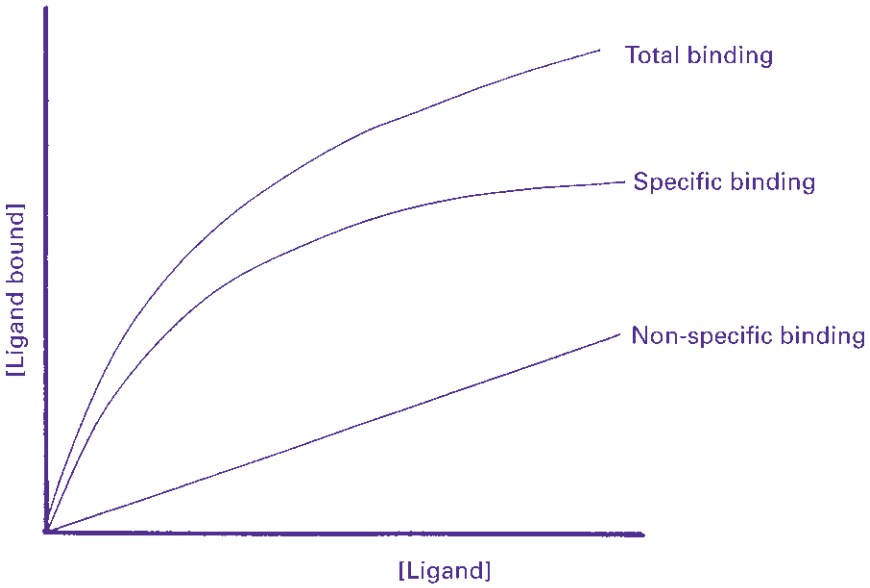


Fig. 16.4. Specific and non-specific binding of a ligand to a membrane receptor. Specific binding is normally hyperbolic and shows saturation. Non-specific binding is linear and is not readily saturated.

number of ways, the simplest of which is centrifugation. Care has to be taken to ensure that the pelleted ligand-bound fraction is washed free from unbound ligand and that in the washing process the dissociation of bound ligand from the receptor is minimised. Alternative methods include equilibrium dialysis and ultrafiltration.

16.3.2 Analytical procedures for the study of receptor–ligand binding

Procedures requiring the separation of bound and free fractions

Equilibrium dialysis The receptor preparation and ligand, each in a buffer of the same pH and concentration, are placed in opposite halves of a dialysis cell. The cell, which generally has a total internal volume of about 3 cm^3, is constructed of transparent plastic such as Perspex^R and unscrews into two halves, which are separated by a cellulose acetate or nitrate semipermeable membrane mounted on an inert mesh support. Many commercial variants of the cell are available, some consisting of banks of up to six cells. The temperature of the cell is thermostatically controlled and the cell is slowly rotated to help the system to reach equilibrium. The ligand molecules, which are small and diffusible, readily cross the membrane until their unbound concentration is the same on both sides (Fig. 16.5). The receptor protein is confined to one half of the cell. At equilibrium, samples are taken from each half of the cell and analysed for ligand. The sample from the receptor half of the cell will give the sum of the bound and unbound ligand concentrations,

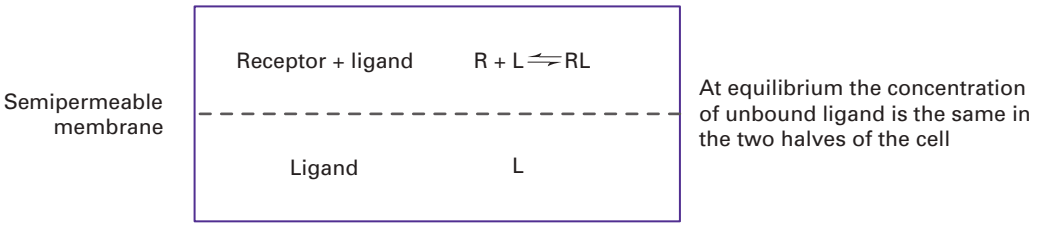


Fig. 16.5. The study of receptor–ligand (R–L) binding by equilibrium dialysis.

whereas that in the other half will simply give the unbound ligand concentration, which will be the same as the unbound ligand concentration in the other half of the cell containing the receptor.

For reliable results the binding of both the ligand and protein to the semipermeable membrane must be minimal and the total ion concentration in each half of the cell equalised to minimise any possibility of a charge inequality on either side of the membrane affecting the distribution of ligand (Donnan effect). The limitations of the technique are the relatively long period of time it takes to establish equilibrium and the fact that it cannot be applied to cases where the ligand is a macromolecule such as insulin, since it will be unable to diffuse across the membrane.

Ultrafiltration The receptor preparation and ligand in a buffered solution are contained in a thermostatically controlled cell (generally 1–3 cm^3 capacity) containing a semipermeable membrane on an inert mesh support at its base. Since no diffusion across a membrane is required to establish equilibrium, attainment of equilibrium is rapid (a few minutes, but this should be checked experimentally). A small sample (100 mm^3) is then forced across the membrane into a collection cup, either by application of a gas pressure to the mixture side or more simply by placing the cell in a low speed centrifuge and centrifuging at about 3000 ***g*** for a few minutes. By analysing the ultrafiltrate (representative of the unbound ligand concentration) and the reaction mixture (bound plus unbound ligand), the influence of ligand concentration on the extent of binding can be studied readily. The attraction of the method is its speed, but binding of the reactants to the semipermeable membrane must be checked and the volume of the ultrafiltrate kept to a minimum to minimise any possibility that the sampling procedure displaces the equilibrium. As with equilibrium dialysis, the method cannot be used to study the binding of macromolecular ligands.

Binding study techniques not requiring the separation of bound and free ligand fractions

Scintillation Proximity Assay™ In this technique the receptor within a membrane fragment is covalently attached to scintillant beads and the ligand is labelled with a weak β-particle emitter such as ^3H or ^{125}I. The reactants are mixed in a photometric cuvette. When the ligand binds to the receptor the β-particles cause the scintillant to emit light that is detected by a photomultiplier and quantified,

thereby allowing the bound ligand to be measured. Unbound ligand distant from the surface of the scintillant beads does not cause light emission, since the low energy β-particles emitted by the unbound ligand are absorbed by the surrounding reaction medium (see Section 14.2.2). Binding can be monitored continuously.

Fluorescence spectroscopy Recently, the use of fluorescence-based techniques for the study of ligand–receptor binding has gained in popularity, as the techniques are ultrasensitive, avoid the need to separate bound and free ligand fractions and are capable of studying binding involving a few or even individual ligand molecules and single receptors. The general principles of fluorescence spectroscopy are discussed in Chapter 12. The methods are based on either the intrinsic fluorescence of the receptor protein or the fact that the ligand or the receptor protein is tagged with a suitable fluorescent marker (fluor). Tagging is generally achieved by chemical attachment to functional groups such as amines, thiols, carboxyls and alcohols. Commonly used fluors include fluorescein, rhodamine and the dye Fluo-3. Alternatively, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* or the red fluorescent protein of *Discosoma striata* can be attached to receptor proteins by genetic engineering techniques without altering the normal function of the protein. The main advantage of using either of these two autofluorescent proteins is that no cofactors are required for fluorescence to occur, hence the study protocols are relatively simple. The intrinsic fluorescence of proteins is linked to the presence of tryptophan residues, which, being hydrophobic, are commonly folded within the internal core of the protein. In all cases the intensity of fluorescence, which may be due to a modification of an amino acid at the active site, not the tryptophan per se, is sensitive to the chemical environment of the fluor so that such small conformational changes induced by ligand binding may result in:

- an increase or a decrease in the intensity of the fluorescence (and hence the quantum yield, Q) owing to changes in quenching as a result of ligand binding;
- a change in the fluorescence spectrum, i.e. a move to a longer or shorter wavelength;
- a change in the fluorescence lifetime, T;
- a change in the sensitivity of the fluor to induction of fluorescence by plane-polarised light.

In the study of the binding of ligands to receptor proteins, measurements may be made on a steady-state (single measurement) basis or a time-resolved (multiple measurements over a period of time) basis most commonly by stopped-flow or quench-flow procedures (Section 15.2.3).

Steady-state fluorescence spectroscopy measurements are based on one of three physical effects:

- Changes in the spectroscopic properties (extinction coefficient, emission wavelength) of the fluor induced by changes in its microenvironment caused by ligand binding and/or an alteration of the diffusion of the receptor protein in the membrane as a direct result of ligand binding. Changes in the intensity

of fluorescence (ΔF) are used as a measurement of the extent of ligand binding, studied as a function of ligand concentration and used in Scatchard or Lineweaver–Burk plots for the calculation of K_d values.

- The induction of fluorescence anisotropy (anisotropy is the directional variation in optical properties (in this case fluorescence) along perpendicular and parallel axes): in this technique fluorescence is induced by plane-polarised (blue) light. Molecules of the fluor orientated parallel to this plane of polarisation will be excited preferentially. However, if some of these molecules rotate after the absorption of the light but before the fluorescence has time to occur, some of the resulting fluorescence will be depolarised (i.e. no longer in one plane). The extent to which this occurs can be used to deduce information about the size, shape and flexibility of the protein carrying the fluor. It can also be used to monitor the binding of a ligand to the protein. The fluorescence intensity is measured parallel (i.e. in the same plane) to the absorbed plane-polarised light and at right angles to it. From the two measurements it is possible to calculate the degree of fluorescence depolarisation and hence the fluorescence anisotropy, both of which are expressed in terms of the difference between the fluorescence parallel to the absorbed plane-polarised light and that perpendicular to it, the difference being expressed as a function of the sum of the fluorescence in the two planes.
- The induction of fluorescence resonance energy transfer (FRET). This technique relies on the presence of two fluors (intrinsic or extrinsic) in distinct locations within the receptor protein such that the emission spectrum of one and the excitation spectrum of the other overlap. In such circumstances it is possible for the emission light of one fluor to be absorbed by the second and be emitted as part of its emission. This process is called resonance energy transfer and the extent to which it occurs is proportional to $1/R^6$, where R is the distance between the two fluors. Ligand binding to the receptor may induce conformational changes that cause R to change.

Time-resolved fluorescence spectroscopy (TRFS) is based on the use of a europium-linked fluor and allows the study of the lifetimes (milliseconds to minutes) of the excited states of the fluor. It can distinguish between free and bound ligand and can give valuable information on conformational changes in the protein induced by the binding of the ligand and on the process of dimerisation of receptor proteins (see Sections 16.5.2 and 16.5.3) both *in vivo* and *in vitro*. It is increasingly popular in the technique of high throughput screening for genomics and proteomics (Section 8.5).

One of the major limitations of many of the techniques commonly used to study receptor–ligand binding is that they can be used to study only the final equilibrium position and are therefore not suitable for studying the rate constants for the formation and dissociation of the receptor–ligand complex. This disadvantage, when coupled with the fact that many of the methods are also slow and not amenable to automation, makes them unattractive for routine, repetitive studies of the kinetics of receptor–ligand binding. Recent developments in gene cloning

have opened up the possibility of cloned receptors, including those of unknown physiological function, being available to the pharmaceutical industry as potential targets for new therapeutic agents. This has stimulated the development of automated techniques for the study of biomolecular interactions without the use of fluorescent or radioisotope labels, with the consequence that dedicated instruments are now available commercially for these types of study, the four most important of which will now be discussed.

Flow cytometry This is a technique for making measurements on individual cells as they flow passed an array of detectors. Most commonly the measurements are based on fluorescence using the principles discussed previously. The technique was originally developed to allow the separation of a mixed population of cells but has been adapted to allow the study of many aspects of receptor function, including receptor–ligand interactions on cell membrane surfaces. It has the great advantage that the measurements are made on individual cells at rates that can be as high as 10 000 s^{-1}, which enables accurate kinetic binding data to be calculated. There are two basic designs of cytometer. In coaxial mixing mode, a process known as hydrodynamic focusing is used to deliver the reactants to the detection point in the cytometer. Separate streams of reactants are brought into coaxial flow by means of a flow nozzle as they enter the cytometer, which is essentially a flow cell. The streams of reactants through the cytometer are surrounded by a faster moving 'sheath' of buffered medium that ensures that the cells flow in single file and such that the flow is not turbulent. The reactants in the coaxial flow mix by diffusion. Alternatively, the reactants are delivered from syringes by computer-controlled stepping motors, similar to those used in the stopped-flow technique for the study of enzyme–substrate interactions (Section 15.2.3), into a mixing chamber and then delivered into the cytometer by an additional syringe and again the flow of mixed reactants through the cytometer is surrounded by a sheath of buffered medium. The cytometer contains one or more light sources, usually lasers of a given wavelength chosen to cause the probe attached to the ligand to fluoresce, arranged at 90° to the flow and focused on the flow of cells. A series of lenses, filters and photomultipliers allow the fluorescence and scattered light to be studied at 90° and 180° to the light source within 100 ms of mixing. Variation of the point of measurement allows the mixing time to be varied and variation of the concentration of the ligand allows kinetic data to be calculated. The technique has also been used to study receptor numbers, desensitisation, interaction with G-proteins and arrestins and internalisation. It is possible to divide the cell flow into droplets after their measurement and to collect cells of a given type by causing appropriate droplets to be electrostatically charged and deflected into a fraction collector. This forms the basis of the technique called fluorescence-activated cell sorting (FACS).

Surface plasmon resonance (SPR) spectroscopy In this technique the receptor under study is immobilised to a sensor 'chip' surface, such as a hydrogel layer on a glass slide, via either biotin–avidin interactions or covalent coupling using amine or thiol reagents similar to those for in affinity chromatography (Section 11.8.2)

(Fig. 16.6a). A typical surface concentration of receptor of 1–5 ng mm^{-2} is used. The sensor chip forms one wall of a micro-flow cell so that an aqueous solution of the ligand can be pumped at a continuous, pulse-free rate across the surface of the immobilised receptor (Fig. 16.6b). This ensures that the concentration of ligand at the surface is maintained at a constant value, which can be varied by altering the concentration of the circulating ligand. Variables such as temperature, pH and ionic concentration are carefully controlled, as is the duration of exposure of the receptor to the ligand. Replacing the ligand solution by a buffer solution enables the dissociation of bound ligand to be studied.

The binding of the ligand to the receptor causes an increase in mass at the surface of the chip. Equally, dissociation of the ligand causes a reduction in mass at the surface. These mass changes in turn cause changes in the refractive index of the medium at the surface of the chip and it is this change in refractive index that is measured, since its value determines the propagation velocity of electromagnetic radiation in that medium. The measurement is based on a phenomenon called surface plasmon resonance (SPR) and on the principle of total internal reflection (TIR) of light. (Plasmon is a term for a collection of conduction electrons in a metal or semiconductor.) When polarised light is totally internally reflected at an interface separating two media with different refractive indices, the reflected beam leaks an electrical field, called an evanescent field wave, into the medium of lower refractive index (in this case the liquid containing the receptor) where it decays at an exponential rate and effectively only travels one wave length. If a thin layer of gold is placed at the interface between the two media, its electron cloud can be made to resonate (hence 'resonance' spectroscopy) by interaction with the plane-polarised component of the evanescent field wave. This SPR enhances the evanescent field wave (Fig. 16.6b) and simultaneously causes a decrease in the intensity of the reflected plane polarised light (Fig. 16.6b). The angle of the incident beam at which this SPR occurs depends on several factors, one of which is the refractive index into which the evanescent wave is propagated. As previously explained, binding of the ligand to the receptor causes a change in refractive index, hence monitoring the angle at which SPR occurs gives a continuous real-time record of receptor–ligand binding.

A wedge-shaped beam of plane-polarised light angled to the surface of the bound receptor is used (Fig. 16.6b) and an array of diode detectors monitors the reflected light. By continuously monitoring the SPR angle as a function of time and measuring the resonance signal (the 'sensorgram' in Fig. 16.6b,c), and by appropriate calibration, the measurements can be used to calculate binding constants and rate constants for the receptor–ligand interactions. The SPR angle is expressed in resonance units (RU) such that 1000 RU corresponds to a change in mass at the surface of the chip of about 1 ng mm^{-2}. The advantages of the technique are that it does not require the molecules to be fluorescent or radiolabelled, it can be used to study molecules as small as 100 Da and can be used with coloured or opaque solutions. In addition to enabling the kinetics of receptor–ligand interactions to be studied, the technique has also been used to study antibody–antigen and protein–protein interactions, identify DNA damage and cell signalling. It is extensively used in

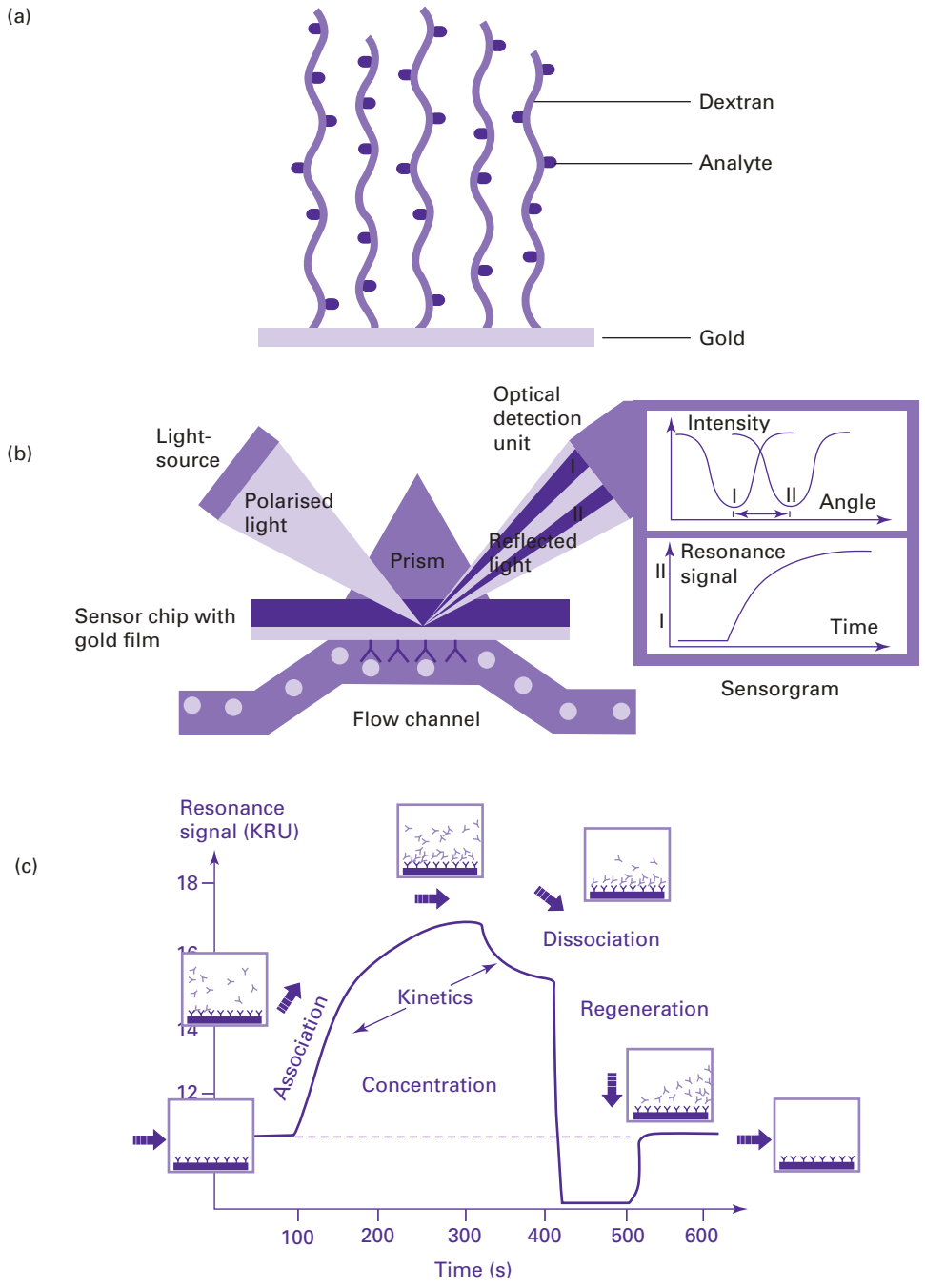


Fig. 16.6. The principles of surface plasmon resonance technology. (a) The sensor 'chip' surface. (b) The flow channel and (insert) change in intensity of reflected light as a function of angle of incidence of the light beam and change in resonance signal as a function of time. (c) The sensorgram. (Reproduced by permission of Biacore AB, Uppsala, Sweden: website <www.biacore.com>.)

proteomic research and in drug discovery and development. It is now possible to couple the technique to MALDI–TOF mass spectrometry (Section 8.5).

Plasmon-waveguide resonance (PWR) spectroscopy is closely related to SPR spectroscopy. In this technique the gold or silver layer is coated with a thicker layer of silica that behaves as a waveguide (a device for propagating electromagnetic radiation). The advantage of this is that the resonances from the metal can be induced by both *p*-polarised light, in which the electric vectors are polarised perpendicular to the resonator surface, and *s*-polarised light in which the electric vectors are polarised parallel to the resonator surface. This allows the anisotropic properties of the molecules near the surface of the silica layer to be studied and this in turn allows information about conformational changes induced in these molecules, for example as a consequence of ligand binding, to be deduced. It is more sensitive than SPR spectroscopy and can be used to study conformational changes in lipid bilayers. Thus a single lipid bilayer is deposited on the resonator surface and the receptor protein inserted from a detergent-solubilised solution. A solution containing the ligand is then passed over the layer, allowing the binding process to be studied. Such studies with G-protein-coupled receptors have shown that agonists, inverse agonists and antagonists can readily be distinguished by the conformational changes they induce in the membrane. Specifically, agonists and inverse agonists increase membrane thickness (agonists more so than inverse agonists) by causing an elongation of the receptor whereas antagonists cause no change (see Section 16.2.1).

Protein microarray technology This approach to the study of receptor–ligand binding is based on the principle that assay systems that use a small amount of capture molecule (the ligand) and a small amount of target molecule (the receptor) can be more sensitive than systems that use a hundred times more material. In this miniaturisation approach, the ligand is immobilised onto a small area of a solid phase, commonly a derivatised glass slide. The resulting 'microspot' contains a high density (concentration) of ligand but a very small amount of it. It is then incubated with the receptor, commonly fluorescently tagged, resulting in the binding of some of the target molecules. Since the microspot covers a small area there is effectively no change in the concentration of the target molecules in the sample even if its concentration was low and the binding affinity was high. This is true provided that $<0.1K_d$ of the capture molecules are bound in the complex, where K_d is the dissociation constant for the complex. The capture–target complex is then quantified by fluorimetric methods and the procedure repeated for a series of increasing microspot sizes (increasing ligand concentration) but such that the density of the capture molecules is constant. If, after each incubation, excess receptor protein is washed away, the remaining complex can be analysed by surface-enhanced laser desorption and ionisation (SELDI) mass spectrometry. In practice, microspots are immobilised in rows on the solid support, allowing the simultaneous analysis of hundreds or even thousands of samples.

Patch clamping This technique is of particular value in the study of kinetics and structure of receptors involved in the ligand or voltage-induced gating of ion

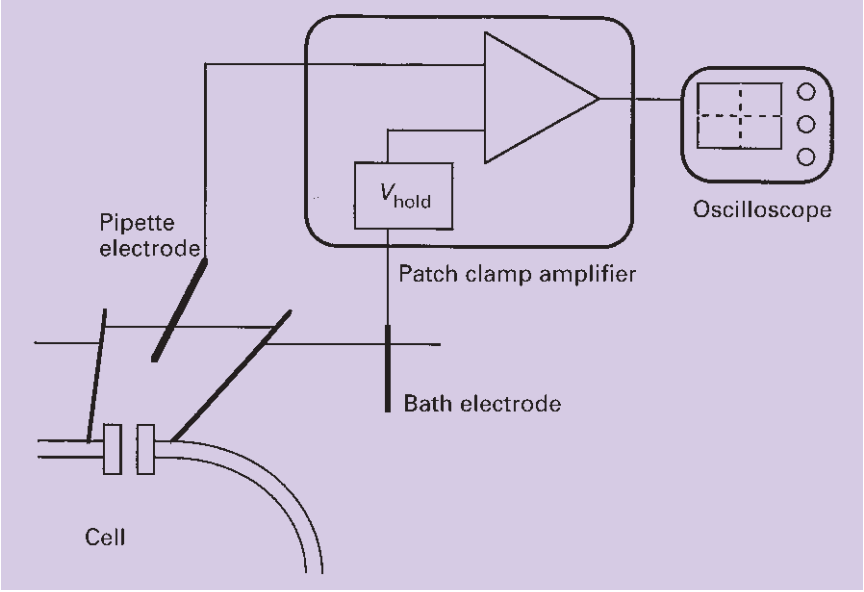


Fig. 16.7. Diagram for a patch clamp set-up in voltage mode. The potential between a bath electrode and a pipette electrode is compared with a reference potential V_{hold}. Current is injected via a feedback circuit until the two potentials are equal. Ion flow across the patch membrane is therefore represented by the required current injected to maintain V_{hold} across the two electrodes. (Reproduced by permission of A. Molleman, University of Hertfordshire.)

movements across the membrane. It involves the use of a glass micropipette with a tip internal diameter of the order of a few micrometres containing a Ag/AgCl electrode and Ringer's solution and, where appropriate, the ligand under study. The tip of the micropipette is brought into contact with the membrane of the cell and a slight vacuum applied. The tip forms a very high resistance seal (10^9 ohms, hence 'gigaohm' seal) with the membrane, thus isolating a patch of about 50 μm^2. The location of the tip is viewed through a microscope. The salt solution in the electrode is connected through the Ag/AgCl junction to a device that allows the simultaneous recording of current and the control of potential (voltage clamp) or vice versa (current clamp) over the patch of membrane (Fig. 16.7). The former option is used more frequently than the latter because the activity of many ion channels is dependent on the potential across the membrane. If the patch contains one or only a few ion channels, ionic currents through the individual channels can be recorded. The magnitude of these currents is of the order of a few picoamperes (10^{-12} A) and they last for a few microseconds. Single-channel traces show the characteristic irregular rectangular jumps representing transitions between the open and closed states of the ion channel (Fig. 16.8). One of the great powers of the technique is that many configurations are possible (Fig. 16.9) each having its distinct advantages depending upon the objectives of the study. Thus the outside-out patch is most convenient

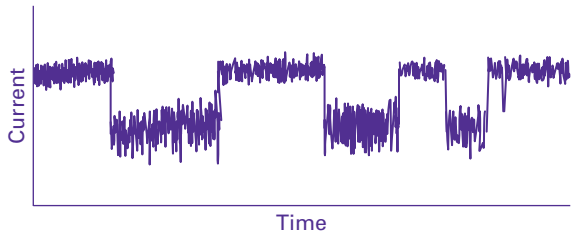


Fig. 16.8. An example of single-channel activity as it appears on the oscilloscope. The current level jumps between two average values as the channel opens (lower level) and closes (upper level). The current scale is of the order of a few picoamperes and the time scale in the range milliseconds to seconds, depending on the channel type. (Reproduced from A. Molleman (2003), *Patch Clamping: An Introductory Guide To Patch Clamp Electrophysiology*, p. 111, by permission of John Wiley and Sons Ltd.)

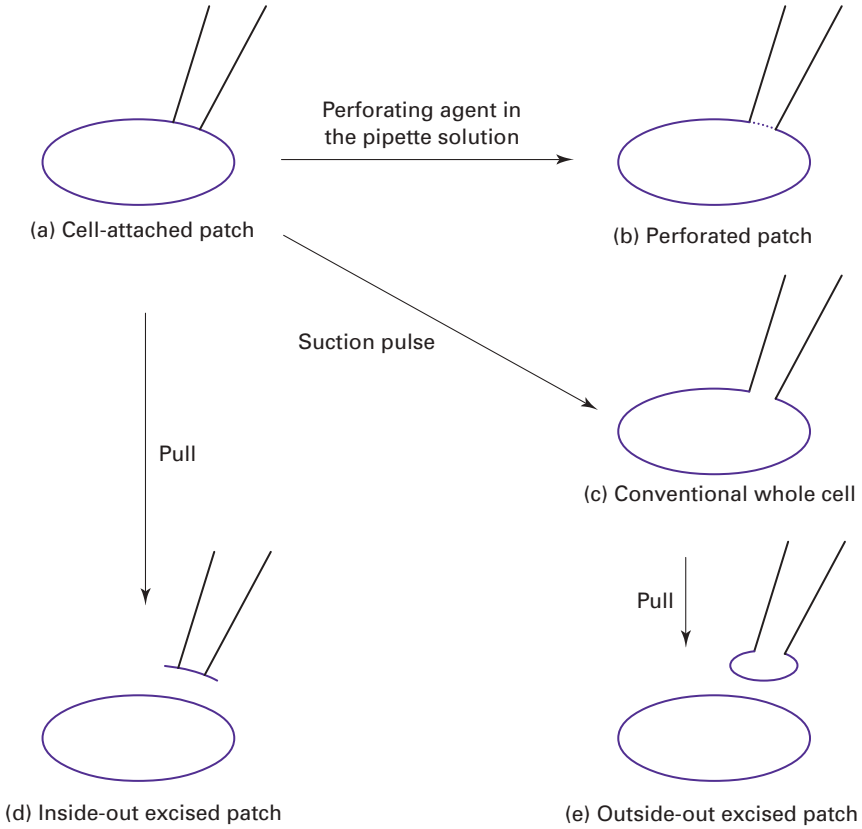


Fig. 16.9. Patch clamp configurations. In (b), (c) and (e) the medium on the extracellular side of the membrane under study can be changed during a recording. In configuration (d) the intracellular side can be manipulated. Configurations (a) and (b) leave the cytoplasm relatively intact. (Reproduced by permission of Areles Molleman, University of Hertfordshire.)

for the application of a range of ligands to the membrane. It is also possible to study the action of a single molecule in *situ*.

By studying the size and direction of the current as a function of ion concentration gradient, deductions can be made about the identity of the ions flowing through the channel. For example, a current of 1.0 pA through a channel for 1 ms is equivalent to the movement of about 7800 sodium ions per channel per millisecond. Patch clamp studies have revealed that the number of ion channels per cell membrane is low.

Example 1 ANALYSIS OF LIGAND BINDING DATA

Question

The extent of the binding of an agonist to its membrane-bound receptor on intact cells was studied as a function of ligand concentration in the absence and presence of a large excess of unlabelled competitive antagonist. In all cases the extent of total ligand binding was such that there was no significant change in the total ligand concentration. What quantitative information about the binding of the ligand to the receptor can be deduced from this data?

	[Ligand] (nM)							
	40	60	80	120	200	500	1000	2000
Total ligand bound (pmol $(10^6$ cells$)^{-1}$)	0.284	0.365	0.421	0.547	0.756	1.269	2.147	2.190
Ligand binding in presence of competitive antagonist (pmol $(10^6$ cells$)^{-1}$)	0.054	0.068	0.084	0.142	0.243	0.621	1.447	1.460

Answer

To address this problem it is first necessary to calculate the specific binding of the ligand to the receptor (B_s). The use of a large excess of unlabelled competitive antagonist enables the non-specific binding to be measured. The difference between this and the total binding gives the specific binding. Once this is known, various graphical options are open to evaluate the data. The simplest is a plot of the specific ligand binding as a function of the total ligand binding. More accurate methods are those based on linear plots such as a Scatchard plot (equation 16.4) and a Lineweaver–Burk plot (equation 16.7). In addition, it is possible to carry out a Hill plot (equation 16.9) to obtain an estimate of the Hill constant, *h*. The derived data for each of these three plots are shown in the following table:

	[Ligand](nM)							
	40	60	80	120	200	500	1000	2000
Total bound ligand (pmol $(10^6$ cells$)^{-1}$)	0.284	0.365	0.421	0.547	0.756	1.269	2.147	2.190
Non-specific binding (B_{ns}) (pmol $(10^6$ cells$)^{-1}$)	0.054	0.068	0.084	0.142	0.243	0.621	1.447	1.460

Example 1 (*Cont.*)

	[Ligand](nM)							
	40	60	80	120	200	500	1000	2000
Specific binding (B_s) (pmol (10^6 cells)$^{-1}$)	0.230	0.297	0.337	0.405	0.513	0.648	0.700	0.730
B_s/[L] $\times 10^3$ (dm^3 (10^6 cells)$^{-1}$)	5.75	4.95	4.21	3.37	2.56	1.30	0.70	0.43
1/[B_s] (pmol (10^6 cells)$^{-1}$)	4.35	3.37	2.97	2.47	1.95	1.54	1.43	1.37
1/[L] (nM)$^{-1}$	0.0250	0.0170	0.0125	0.0083	0.0050	0.0020	0.0010	0.0005
($B_{max} - B_s$) (pmol (10^6 cells)$^{-1}$)	0.52	0.45	0.413	0.345	0.237	0.102	0.050	0.020
B_s/($B_{max} - B_s$)	0.44	0.66	0.816	1.174	2.164	6.35	14.00	36.50
log B_s/($B_{max} - B_s$)	−0.356	−0.180	−0.088	0.070	0.335	0.803	1.146	1.562
log [L]	1.60	1.78	1.90	2.08	2.30	2.70	3.00	3.30

The hyperbolic plot allows an estimate to be made of the maximum ligand binding, B_{max}. It is approximately 0.75 pmol (10^6cells)$^{-1}$. An estimate can then be made of K_d by reading the value of [L] that gives a ligand binding value of $0.5B_{max}$ (0.375 pmol (10^6 cells)$^{-1}$). It gives an approximate value for K_d of 100 nM.

A Scatchard plot obtained by regression analysis gives a correlation coefficient, r, of 0.996, B_{max} of 0.786 pmol (10^6 cells)$^{-1}$ and K_d of 97.3 nM. A Lineweaver–Burk plot gives a correlation coefficient, r, of 0.998, B_{max} of 0.746 pmol (10^6 cells)$^{-1}$ and K_d of 90.5 nM. Note that there is some variation between these three sets of calculated values and the ones given by the Lineweaver–Burk plot are more likely to be correct since, as previously pointed out, the Scatchard plot overestimates both values when the binding data are subjected to linear regression analysis.

The Hill plot based on a value of B_{max} of 0.75 pmol (10^6 cells)$^{-1}$, gave a correlation coefficient of 0.998 and a value for the slope of 1.13. This is equal to the Hill constant, h.

16.3.3 Receptor–ligand binding data

K_d, k_{+1}, k_{-1} values and receptor numbers and occupancy

The K_d values observed for a variety of receptors binding to their physiological agonist are in the range 10^{-6} to 10^{-11} M, which is indicative of a higher affinity than is typical of enzymes for their substrates. The corresponding k_{+1} rate constants are in the range 10^5 to 10^8 (M)$^{-1}$ min^{-1} and k_{-1} in the range 0.001–0.5 min^{-1}. Studies with G-protein-coupled receptors that form a tertiary complex (AR*G) have shown that the tertiary complex has a higher affinity for the ligand than has the binary complex (AR*). Receptor affinity for its agonist is also influenced by

receptor interaction with various adaptor protein molecules present in the intracellular cell membrane. This is discussed more fully later.

It is relatively easy to calculate the number of receptors on cell membranes from binding data. The number is in the range 10^3–10^6 per cell. Although this may appear large, it actually represents a small fraction of the total membrane protein. This partly explains why receptor proteins are sometimes difficult to purify. From knowledge of receptor numbers and the K_d values for the ligand, it is possible to calculate the occupancy of these receptors under normal physiological concentrations of the agonist. In turn it is possible to calculate how the occupancy and the associated cellular response will respond to changes in the circulating concentration of the agonist. The percentage response change will be greater the lower the normal occupancy of the receptors. This is seen from the shape of the dose–response curve within the physiological range of the agonist concentration. It is clear that, if the normal occupancy is high, the response to change in agonist concentration is small. Under such conditions, the response is likely to be larger if the receptor–agonist binding is a positively cooperative process.

Binding studies have revealed that some agonists can stimulate the maximal response from the receptor preparation with only a small proportion of the receptors occupied. This has given rise to the concept of spare receptors, which are indistinguishable from the occupied receptors. It is believed that spare receptors maximise the sensitivity of the cell to the available agonist. However, to regard 'spare' receptors as being in excess of physiological requirement is misleading and in this sense the term is unfortunate. 'Spare' receptors are an intrinsic part of the cell's strategy to ensure a maximum response to a low dose of agonist and to facilitate speed in on–off responses. However, binding studies have also shown that the number of receptors in a membrane is time variable. The number is subject to upregulation or downregulation, depending upon the needs of the cell. Control of receptor numbers is exerted at a number of levels ranging from transcription and translation to endocytotic internalisation and subsequent degradation by ubiquitin-linked enzymes (Section 16.6.2). Some receptors can be shown by labelling studies to be recycled back into the membrane following endocytosis, the whole cycling time taking as little as a few minutes. The half-life of the insulin receptor is 9 h. Prolonged exposure of receptors to their ligand has been shown to result in desensitisation in some cases. The mechanism of this process is discussed later (Section 16.6.1).

Receptor subclasses

Binding studies using both agonists and antagonists have identified the heterogeneity of many membrane receptors. Classification of receptor subclasses based on such studies is not without ambiguity, and indeed controversy, and clearly identifies the need for such classification studies to be paralleled by those based on a gene cloning approach. As an example, this dual approach has confirmed the existence of over a dozen types of 5-HT (5-hydroxytryptamine or serotonin) receptor. An interesting feature of some receptor subclasses is that not only do they have different binding characteristics but they may also trigger opposing cellular

responses. As an example, β-adrenergic receptors are activated by adrenaline and noradrenaline but there are three subclasses β_1, β_2 and β_3 with different amino acid composition, affinities for agonists and physiological responses. Thus β_1-adrenergic receptors mediate cardiac responses, β_2-adrenergic receptors are involved in skeletal and smooth muscle function, and β_3-adrenergic receptors are involved in metabolic responses. Subtype-selective synthetic agonists such as salbutamol readily discriminate between the three groups.

Receptor mobility

The ability of receptors to interact with other membrane-associated molecules, essential for the process of signal transduction, requires the receptor to diffuse freely within the two-dimensional lipid bilayer. This receptor mobility can be studied by the technique of fluorescence recovery after photobleaching (FRAP) that requires the receptor to be tagged with a fluor. Labelled receptors within a small circular region of the membrane (1–10 μm^2) are then exposed to an intense attenuated laser beam that irreversibly bleaches the fluorescent receptors within the beam. The subsequent time-dependent increase in fluorescence within this bleached area, owing to diffusion into the area of non-bleached fluorescent receptor molecules, is then monitored, allowing the rate of diffusion of the receptors to be calculated. Such studies have revealed that a given receptor can traverse the whole surface of a cell in times ranging from 4 min to 7 h.

16.4 MOLECULAR STRUCTURE OF RECEPTORS

16.4.1 Molecular nature of the receptor

In principle, the study of the molecular nature of a receptor protein and its linkage to signal transduction is best carried out with the receptor embedded in the membrane. However, low expression levels and the presence of unrelated proteins within the membrane can complicate such studies. In spite of these difficulties, it is possible to study receptor proteins in their membrane environment either in the native state or by using purified receptor protein inserted into liposomes (artificial membrane structures) or giant unilamellar vesicles (diameter up to 100 μm). The techniques employed for such studies include the following:

- *Freeze-fracture electron microscopy:* The procedure enables the oligomeric nature of the receptor in the membrane to be evaluated and an estimate made of the number and size of transmembrane α-helices.
- *Optical microscopy:* Studies can be made of the rate of diffusion of individual receptor molecules in the fluid membrane.
- *Electron-spin resonance spectroscopy (ESR):* Using a nitroxide spin label attached to a cysteine residue, studies can be made of the effective radius of the protein and its rotation in the membrane.
- *Anti-receptor antibodies:* These have been used to study aspects of receptor biochemistry, including their cellular location and the location of binding

sites within the receptor. Anti-receptor antibodies can be raised using cell-bound or membrane-bound receptor protein, purified receptor protein or receptor protein produced by gene cloning. One of the problems associated with the use of anti-receptor antibodies is that they will certainly have more than one epitope, some of which may be located within the membrane or on the intracellular side of the membrane rather than simply on the ligand-binding side of the membrane. Thus anti-receptor antibodies may bind to sites other than the physiological ligand-binding site and may even trigger receptor aggregation owing to their large multivalent nature. The use of monovalent Fab antibody fragments (Section 7.1.2) may help to confirm whether or not the ligand-binding site is being studied.

It is possible to release the receptor from its membrane by treatment with detergents and to isolate the receptor by conventional techniques especially lectin affinity chromatography, affinity chromatography using an antagonist as the immobilised ligand, or hydrophobic interaction chromatography. The purified protein, in detergent solution, can then be studied by a number of techniques:

- *Analytical ultracentrifugation:* The procedure of sedimentation equilibrium (see Section 3.5.3), which is independent of the shape of the protein, allows a study of the receptor's size and the stoichiometry and interaction between its subunits. The alternative procedure of sedimentation velocity allows an assessment of the purity of the preparation and an estimate of the relative molecular mass of the receptor, provided details of the receptor shape are available. However, the binding of detergent molecules to the protein can complicate the interpretation of the data.
- *Molecular exclusion chromatography:* In principle this technique enables an estimate to be made of the relative molecular mass but the choice of calibration proteins is a potential problem.
- *Gel electrophoresis:* using the Coomassie Brilliant Blue technique, an estimate of purity and the relative molecular mass of the receptor can be made.

Once purified, the receptor protein can be crystallised and its structure investigated by X-ray crystallography. However, it is far more common to undertake such studies on recombinant proteins. The crystal structure of a number of receptor proteins has been studied. Examples include the rhodopsin, glutamate, insulin and nicotinic acetylcholine receptors. The crystal structures have provided an understanding of the molecular mechanisms that are responsible for the physiological function of the receptors. The main limitations of crystallographic studies are the inherent difficulty of producing high quality crystals and the fact that the technique gives data only on the solid phase of the protein.

The main alternative to the X-ray crystallography is NMR spectroscopy especially multidimensional NMR by the COSY, NOESY and ROESY procedures (Section 13.4.1). The advantages of the NMR approach are that it can be applied to the receptor protein in solution and the fact that the more flexible parts of the protein give a stronger NMR signal thus making it ideal for the study of

conformational changes induced by ligand binding. NMR studies of the rate of deuterium exchange in aqueous solution by receptor proteins have been particularly successful in showing that receptor proteins exist as a collection of many microconformations (a so-called protein assembly), some of which are energetically more favourable than others, and that there is constant motion between states with similar energies. These microconformations result from the folding and unfolding of specific small hydrophobic regions of the protein. Indirect evidence for this protein mobility comes from studies with G-protein-coupled receptors. It has been possible to immunoprecipitate complexes between the receptor and a G-protein in the absence of the receptor agonist, confirming that the receptor must be flipping spontaneously between inactive and active states. Binding of an agonist may induce structural changes that facilitate the formation of otherwise energetically less favourable states. Equally, different agonists for a given receptor may induce different states, thus rationalising differences in their efficacy.

16.4.2 Receptor-binding domains

The classical experimental strategies adopted for the study of the binding domain of receptor proteins are very similar to those used so successfully for the study of enzyme active sites. Affinity labelling (Section 15.4.2) has been adopted, with success, for the insulin receptor. The insulin receptor consists of two α-subunits (molecular mass 84 kDa) and two β-subunits (molecular mass 70 kDa) linked as a tetramer by disulphide bridges. Hydrophobicity studies (Section 8.4.3) revealed that only the two β-subunits span the membrane and protrude into the intracellular region. The two α-subunits are entirely on the outside of the membrane. In the study of the insulin-binding site, 4-azido-2-nitrophenyl-^{125}I-insulin was used as a photoaffinity label. Once the label was bound to the insulin receptor, the receptor–label complex was exposed to ultraviolet light, which converted the azido group to a nitrene that covalently attached itself to the binding site. The reagent was shown to be linked to an α-subunit. The N-terminal regions of the α-subunits are cysteine rich and contain a binding domain that has similarities to the ligand-binding site identified for the epidermal growth factor (EGF) receptor.

There are two modern approaches to the study of receptor-binding domains. The first is to study the receptor–ligand specificity by binding studies and to use computer-based molecular modelling analysis of the structure/specificity relationships to deduce the details of the binding domain. The second is to study the crystal structure of the receptor protein in the presence and absence of the physiological ligand. Both approaches can be complemented by site-directed mutagenesis studies that enable key amino acid residues in the binding domain to be identified. Such approaches to the study of the tyrosine kinase receptors have identified 20 subclasses that all have multiple extracellular domains containing common folds that may be cysteine rich, Ig like or fibronectin III like.

The realisation that receptor proteins have considerable mobility and are constantly flipping between different conformations means that the binding domain must be flexible rather than rigid and hence that the domain shape and size is to

some extent determined by the ligand. This explains why, for some receptors, structurally diverse ligands can bind with similar ease.

Classification of cell surface receptors

The application of the various analytical approaches discussed above to the study of structure and size of receptor proteins coupled with studies of the type of transduction mechanism induced by the receptors, has lead to the identification of three main types of cell membrane receptors:

- *Ligand-gated ion-channel receptors:* These are responsible for the selective movement of ions such as Na^+, K^+ and Cl^- across membranes. Binding of the agonist triggers the gating (opening) of the channel and the movement of ions across the membrane. This ion movement is a short-term, fast response that results in the propagation of a membrane potential wave. They may be excitatory and result in the depolarization of the cell (e.g. the nicotinic acetylcholine and ionotropic glutamate receptors), or directly or indirectly inhibitory (e.g. the γ-aminobutyric acid A ($GABA_A$) receptors, which stimulate chloride influx and thus result in the repolarisation of the cell). All receptors in this class consist of four or five homo- or heteromeric subunits. Responses produced by this class of receptors occur in fractions of a second.
- *G-protein-coupled receptors (GPCRs):* Receptors in this class are linked to a G-protein that is trimeric. Receptor activation triggers its interaction with a G-protein, resulting in the exchange of GTP for GDP on one subunit that dissociates from the trimer causing the activation of an effector molecule such as adenylyl cyclase that is part of an intricate network of intracellular signalling pathways. Responses produced by GPCRs occur in a timescale of minutes.
- *Protein kinase receptors:* These receptors all undergo agonist-stimulated autophosphorylation in the intracellular region. This activates the kinase activity towards intracellular proteins. The majority of activated receptor kinases catalyse the transfer of the γ-phosphate of ATP to the hydroxyl group of a tyrosine in the target protein. This phosphorylation process controls the activity of many vital cell processes. Members of a minor subgroup of protein kinase receptors transfer the phosphate group of ATP to a serine or threonine group rather than tyrosine. Examples of protein kinase receptors include the insulin receptor and the EGF receptor. Responses produced by protein kinase receptors occur over a timescale of minutes to hours.

Structural studies coupled with molecular cloning techniques have revealed that all cell membrane receptor proteins possess three distinct domains:

- *Extracellular domain:* This protrudes from the external surface of the membrane and contains all or part of the ligand-binding site.
- *Transmembrane domain:* This is inserted into the phospholipid bilayer of the membrane and may consist of several regions that loop repeatedly across the membrane. In some cases these loops form a channel for the 'gating' (hence

the channel may be open or closed) of ions across the membrane, whilst in other receptors the loops create part of the ligand-binding site.

- *Intracellular domain:* This cytosolic region of the protein has to respond to the extracellular binding of the ligand to initiate the signal transduction process.

The existence of three domains within receptor proteins reflects their amphipathic nature. Each receptor protein has regions of 19–28 amino acid residues that are hydrophobic, consisting of non-polar side-chains, and other regions that are hydrophilic, consisting of polar and ionised side-chains. The hydrophobic regions, generally in the form of α-helices, are the transmembrane regions that are inserted into the non-polar, long-chain fatty acid portion of the phospholipid bilayer of the membrane. In contrast, the hydrophilic regions of the receptor are exposed on the outside and inside of the membrane where they interact with the aqueous, hydrophilic environment. The need for the hydrophobic region to span the membrane is so characteristic of receptors that it is possible to identify the membrane-spanning regions simply by inspecting the amino acid sequence of the protein. Superfamilies of receptor proteins can be recognised from the precise number of transmembrane regions each possess. Such multiple TM regions may be the consequence of the oligomeric nature of the receptor protein and in many cases the function of the receptor is linked to this oligomeric nature (see Section 8.4.3).

Receptor superfamilies

One-pass receptors (one transmembrane domain, 1TM) Members of this group include the insulin receptor, the EGF receptor and the platelet-derived growth factor (PGDF) receptor, each of which has intrinsic protein kinase activity.

Two-pass receptors (2TM) Members of this family have the involvement of ATP in common and as a consequence they are referred to as P2 receptors. There are two subtypes: P2X, which are ligand-gated ion-channels, and P2Y, which are G-protein coupled. In all cases there is one intracellular loop and the N- and C-terminal ends are located extracellularly.

Three-pass receptors (3TM) These receptors are all ligand-gated ion channels involving excitatory amino acid neurotransmitters such as L-glutamate. The best-studied examples are the NMDA (*N*-methyl-D-aspartate) receptor and the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptor. There is one intracellular and one extracellular loop. The N-terminal region is extracellular and the C-terminal region intracellular. All receptors in the class are homo- or heteropolymeric and require the binding of two agonist molecules for activation.

Four-pass receptors (4TM) These receptors are also all ligand-gated ion channels related to the nicotinic acetylcholine receptor, serotonin receptor (5-HT_3), glycine receptor and the γ-aminobutyric acid ($GABA_A$ and $GABA_C$) receptors. In all cases the N- and C-terminal ends are located extracellularly. Only the TM2 region has α-helical structure.

Six-pass receptors (6TM) This is currently a very small receptor family. Two examples, both of which are ligand-gated ion channels, are the inositol trisphosphate (IP_3)-activated receptor (present on the intracellular membrane) and the capsaicin-activated vanilloid receptor. Each receptor has three intracellular and two extracellular loops and both N- and C-terminal ends are extracellular.

Seven-pass receptors (7TM) This is the largest and most diverse receptor superfamily. Many, but not all, are G-protein coupled. The range of first messengers involved with this family includes ions (Ca^{2+}), amino acids (glutamate), amines (catecholamines), purines (ATP), lipids (prostaglandins), peptide hormones (bradykinin), neuropeptides (tachykinins), kinins (interleukin-8) and glycoprotein hormones (thyroid-stimulating hormone, follicle-stimulating hormone). Each receptor has three extracellular and three intracellular loops. The N-terminal region is extracellular and the C-terminal region intracellular.

16.5 MECHANISMS OF SIGNAL TRANSDUCTION

16.5.1 Signal transduction through ligand-gated ion channels

Ligand-gated ion channels constitute one of the mechanisms for the control of the transmembrane movement of ions down their concentration gradient, resulting in a change in membrane potential. This control of ion movement is exerted on the basis of ion type (anion or cation), ion charge and ion size. Binding of the ligand to the resting state of the receptor induces a conformational change in the receptor protein, which results in the opening of the channel and the movement of ions. The channel remains open until either the ligand is removed or when, in the continued presence of the ligand, the receptor protein changes to its desensitised state, in which case the channel is closed. Since this mechanism of transduction is independent of any other membrane component or intracellular molecule, the cellular response to ligand binding is almost instantaneous. This class of membrane receptors includes numerous receptors that are involved in signal transmission between neurones, between glia and neurones, and between neurones and muscles.

Four superfamilies of ligand-gated ion channels, classified on the basis of the number of transmembrane segments within the subunits (2TM, 3TM, 4TM and 6TM), have been identified. The 4TM family has been the most thoroughly investigated and one of its major members is the excitatory nicotinic acetylcholine receptor (nAChR) found in large amounts in the eel and electric ray. Mammalian muscle nAChRs are located on the membrane of the postsynaptic cell adjacent to the synaptic neurone and are involved in muscle contraction. The snake venom toxin α-bungarotoxin binds irreversibly to the receptor, causing a block to the action of acetylcholine. The receptor has been shown to consist of five subunits of four types, α, β, γ and δ, with a stoichiometry of $\alpha_2\beta\gamma\delta$ and a molecular mass of 280 kDa. All of the five subunits span the membrane four times, mainly with α-helical structure, but with some β-structure. Each α-helical region has

been designated TM1–TM4 and the experimental evidence from photoaffinity labelling studies and the production of point mutations supports the view that each of the TM2 regions of the five subunits lines the ion channel with the TM1 and TM3 regions, forming a scaffold to support the channel. Binding studies using native receptors and cloned receptors expressed in fibroblasts have provided evidence for the allosteric binding of two molecules of agonist with a Hill constant (Section 15.3.3) of about 2. Affinity labelling studies using competitive antagonists with a wide range of molecular structures have shown that the ligand-binding sites are in a cleft between two subunits. In both cases an α-subunit has the principal role in binding, with the γ- and δ-subunits playing a minor role. The first ligand molecule binds to the α_1-subunit that is in contact with the δ-subunit and the second molecule to α_2, which is in contact with the γ-subunit. Thus the two binding sites are not structurally identical. Genetically engineered variants of the five subunits structure have shown that the absence of the α-subunit results in the lack of binding of acetylcholine, thereby confirming the importance of this subunit. Mutagenicity and affinity labelling studies using irreversible competitive antagonists have demonstrated the importance to ligand binding of the A, B and C loops within the α-subunits and, in particular, a number of aromatic amino acid residues within these loops. Interestingly, there is high conservation of these residues within the known variants of nAChR.

Electron microscopic study of the nAChR on the *Torpedo torpedo* electric organ postsynaptic membrane has given an indication of the three-dimensional structure of the channel. It is funnel shaped, with a large proportion of the receptor outside the membrane protruding into the postsynaptic cleft. The channel is 25–30 Å wide at the entrance and only 6.4 Å wide at its narrowest point. Three rings of negatively charged amino acid residues, all on the four TM2 helices, line the narrow part of the channel and appear to determine its selectivity. The importance of these amino acid residues has been confirmed by mutagenicity studies.

Patch clamp studies of the nAChR have enabled the kinetics of channel opening to be evaluated. The whole process is kinetically complex but the essential features may be represented as follows:

$$\underset{\text{Channel:}}{} \underset{\text{closed}}{A + R} \rightleftharpoons \underset{\text{closed}}{AR + A} \rightleftharpoons \underset{\text{closed}}{A_2R} \rightleftharpoons \underset{\text{open}}{A_2R^*} \rightleftharpoons \underset{\text{desensitised}}{A_2R^{**}}$$

where A is acetylcholine and R the receptor containing two binding sites for acetylcholine, one on each α-subunit.

Measurement of the numerous rate constants for these reversible processes has revealed that the rate constant for the opening of the channel is greater than the rate constants for the corresponding reverse process (i.e. the reversion to the closed conformation), for the dissociation of a ligand molecule from the closed conformation and for the transition from the open active state to the desensitised state. The consequence of this is that many opening and closing events of the channel occur before either the transition to the desensitised state or a molecule of ligand dissociates from the binding site. The mean channel open time for *Torpedo*

nAChR is 3.0 ms and the mean closed time 94 μs within the bursts of opening and closing activity. The desensitised receptor (R**) eventually reverts to the closed resting state (R).

Desensitisation may be linked to phosphorylation. All five subunits contain amino acid residues located between the TM3 and TM4 regions that are potential sites for phosphorylation, and phosphorylation of the receptor has been shown to occur at two serine residues on each of the γ- and δ-subunits. Each phosphate group introduces two negatively charged oxygen atoms that could induce important conformational changes in the receptor structure and desensitisation. Mutagenesis studies of these serine residues have shown that their replacement by non-polar amino acids minimises the susceptibility of the receptor to acetylcholine-induced desensitisation. In contrast, replacement of the serine residues by glutamate, which contains negatively charged carboxyl groups, permanently desensitises the receptor. Such Glu and Ala substitutions are widely used as a technique in the study of protein kinases. This phosphorylation-induced modulation of receptor function is found in many other types of ion-transport proteins, indicating a common mechanism, but it is not clear whether or not phosphorylation is a prerequisite for receptor desensitisation (Section 16.6.1).

Structural studies similar to those carried out on the nAChR have been carried out on other ligand-gated ion-channels, in particular the $GABA_A$ and glycine receptors. These studies have provided evidence for commonality of channel structure and mechanism of ion selectivity. Consistent with this is the observation that there is considerable conservation of amino acid sequence in specific regions of the respective subunits. However, not all receptors linked to ion channels are directly ligand gated, but rather involve G-proteins as an intermediate between ligand binding and channel gating. An example is the cardiac muscarinic receptor linked to K^+ channels.

16.5.2 Signal transduction through G-protein-coupled receptors

Unlike receptors linked to ion channels of the nAChR type, in which agonist binding is linked directly to channel gating, most receptors require another protein to couple ligand binding to the transduction of the cellular response. In the case of the majority of the seven-pass superfamily (7TM) receptors (see below), the linkage is made by a family of membrane-associated proteins collectively known as G-proteins, so named because they bind GTP and GDP. It has been estimated that the human genome encodes for more than 1000 GPCRs, emphasising their considerable importance. With this group of receptors, the cellular response to agonist binding is linked to the formation of a ternary complex AR*G, although receptors with constitutive activity may act via the binary complex R*G (see Section 16.2.1).

7TM receptor structure

All GPCRs have a tertiary structure that consists of the seven transmembrane helices linked by alternating intracellular and extracellular loops with an

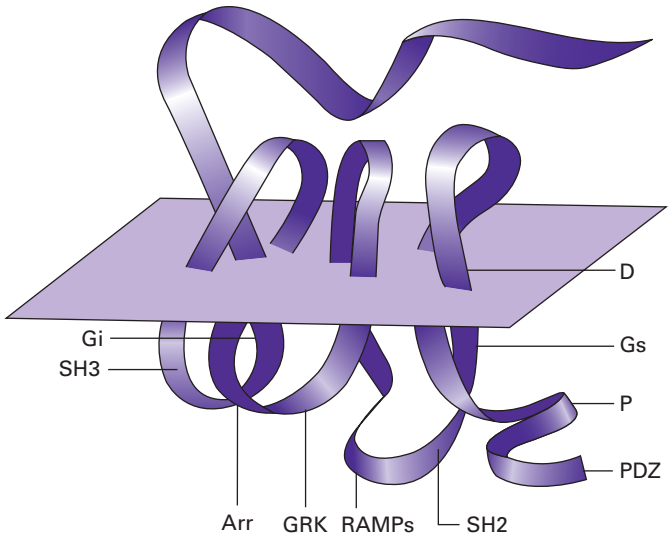


Fig. 16.10. Schematic diagram of a hypothetical G-protein-coupled receptor (GPCR). Labels denote general regions of interaction of the receptor with other cellular proteins including different G-proteins (G_i and G_s), PDZ, SH2- and SH3-domain proteins, receptor-activity-modifying proteins (RAMPs), arrestin (Arr), G-protein-coupled receptor kinase (GRK), sites for dimerisation with other GPCRs (D), and phosphorylation sites that lead to uncoupling and internalisation (P). Any one of these active processes could be considered a form of expression of efficacy. The figure is a general description of various loci for protein interactions, but does not represent accurate locations, as, in most cases, these are not well characterised. (Reproduced from T. Kenakin (2002), Efficacy at G-protein-coupled receptors. *Nature Reviews Drug Discovery*, **1**, 103–110, by permission of Macmillan Magazines Ltd.)

extracellular N-terminal end and an intracellular C-terminal region (Fig. 16.10). Although these receptors have this common 7TM structural feature, their ligand-binding domains vary considerably. For small agonists (e.g. adrenaline, histamine, dopamine, serotonin) the domain is partially embedded within the transmembrane helical structure, but for large agonists, including the neuropeptides and chemokines, the domain may span the extracellular loops or be located near the N-terminal region. This variability in the location of the binding domain emphasises that there must be several ways by which the agonist can stabilise the active conformation. The C-terminal region contains key proline-containing domains by which the active receptor couples with the G-protein.

GPCRs have been classified into three families:

- *Family A (also called class I):* This is characterised by the presence of several conserved regions in the TM loops and the presence of a palmitolyated cysteine in the C-terminal tail. There is 15–20% homology between all the members. The family includes the rhodopsin, adrenergic, histamine, dopaminergic, muscarinic and tachykinin receptors. It is the largest group.

- *Family B (class II):* This is characterised by a long N-terminal tail containing six conserved cysteine residues linked by disulphide bridges and involved in ligand binding. The family includes the glucagon, calcitonin, secretin and parathyroid hormone receptors. They all activate adenylyl cyclase and couple through the same G_s-protein.
- *Family C (class III):* These members bind either glutamate or GABA or are involved in either Ca^{2+} metabolism or taste. Members include some of the metabotropic glutamate receptors (mGluR), of which eight have been identified and each shown to be distributed in specific regions of the brain. Binding of glutamate triggers the production of a second messenger that modulates the activity of the direct-gated (ionotropic) glutamate receptor. mGluRs contain an allosteric site that is a potential target for drugs involved in the treatment of Parkinson's disease (mGluR4), schizophrenia (mGluR5) and addiction (mGluR2).

There is considerable experimental evidence to indicate that GPCRs interact to form homo- and heterodimers and oligomers. In the case of the receptors in families B and C the evidence is that the functional activity of the receptors is linked to these forms, but in the case of family A receptors this link is less clear. The functional unit of both the mGluR1 and $GABA_B$ receptors in family C, for example, is a homodimer. X-ray crystallographic studies on the glutamate receptor have indicated that the dimer exists as a dynamic equilibrium between two conformations, one 'open' and the other 'closed', and that the role of glutamate is to stabilise the closed, active form. In contrast, the $GABA_B$ receptor is a heterodimer involving two receptors from family B.

The molecular activity and specificity of family B receptors is linked to the formation of both dimers and oligomers. In some cases the oligomeric association involves receptor–receptor interaction but in others it involves the association of the monomeric receptor with a member of a family of receptor activity-modifying proteins (RAMPs). Currently three RAMPs (RAMP1, 2 and 3) have been characterised and shown to be relatively small (RAMP1 is a 140 amino acid residue protein) with a single membrane-spanning domain, a large extracellular domain and a small intracellular domain. The RAMP–receptor heterodimers determine the specificity of the functional protein. Thus association of RAMP1 with calcitonin-receptor-like (CL) receptor gives a high affinity calcitonin gene-related peptide (CGRP) receptor whereas interaction of the CL receptor with either RAMP2 or 3 gives rise to an adrenomedullin receptor. Confocal microscopic evidence indicates that these heterodimers are formed in the endoplasmic reticulum and the Golgi apparatus and are retained during the processes of exocytosis to the membrane surface, ligand binding, endocytosis and ubiquitin-directed degradation (Section 16.6.2).

Receptor–signal transduction protein complexes

For the majority of the time GPCRs exist in the cell membrane as complexes with signal transduction proteins rather than as free floating proteins. These

complexes are stabilised by scaffolding or adaptor proteins whose functions are similar but different in detail. Examples of the adaptor proteins include the multi-PDZ proteins, the Shank family of proteins and the Homer proteins. As their name implies, multi-PDZ proteins possess a number of PDZ (*P*SD-95, *D*ig and *Z*O-1/2) domains each of which can bind to the C-terminal region of different receptor and effector proteins involved in the transduction of a given signal. The resulting complex ensures speed and efficiency of the propagation of the signal, which is especially important in synaptic transmission. The Shank proteins possess several protein–protein interaction motifs including the SH2 (*S*rc-*h*omology domain 2) motif that recognises and binds tyrosine-phosphorylated sequences (this includes some receptors with intrinsic protein kinase activity (Section 16.5.3) and the SH3 (Src-homology domain 3) motif that recognises and binds sequences that are proline rich. GPCRs therefore possess domains capable of recognising and binding to these various motifs (Fig. 16.10). They also appear to possess an allosteric regulatory site. In the majority of cases the identity of the modulator is unknown but the site is a potential target for new therapeutic drugs.

G-protein structure

G-proteins are heterotrimeric, consisting of one of each of three subunits: α (40–45 kDa), β (36–40 kDa) and γ (8 kDa), which are loosely attached to the inner surface of the cellular membrane through lipophilic tails on the α- and γ-subunits. The β- and γ-subunits are firmly attached to each other but the linkage to the α-subunit is weaker. In addition to the binding site for the β-subunit, the α-subunit has a binding site for the C-terminal region of the receptor located near the N-terminal end and a guanine nucleotide-binding site that also possesses GTPase activity. Although the α-subunit contains the binding site for the receptor, binding occurs only when the α-subunit is bound to the βγ-dimer. Studies have revealed a very complex picture of the G-proteins. Twenty different α-subunits, 6 β-subunits and 12 γ-subunits have been identified. The potential number of different Gαβγ functional trimers is therefore very large.

G-protein subgroups

Eight subgroups of G-proteins have been classified on the basis of their action. The five most important are as follows:

- the G_s subgroup that stimulates adenylyl cyclase,
- the G_i subgroup that inhibits adenylyl cyclase and activates some K^+ channels and phosphotidylinositol 4,5-bisphosphate (PIP_2) hydrolysis,
- the G_q subgroup that couples receptors to calcium mobilisation through phospholipase C_β, which generates the two second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG),
- the G_0 subgroup that reduces the probability of opening of some voltage-gated Ca^{2+} channels involved in neurotransmitter release,
- the G_t subgroup that stimulates phosphodiesterase following light stimulation of rhodopsin.

The study of G-protein transduction

Numerous methods have been used to study the structure of G-proteins and their interaction with receptors and intracellular effectors. Examples include the following:

- Use of analogues of GTP that bind to the α-subunit but are poorly hydrolysed, effectively leaving the α-subunit permanently active, e.g. [^{35}S]GTPγS.
- Use of bacterial toxins, such as cholera and pertussis toxin, which act by virtue of the fact that they are ADP-ribosyl transferases and stimulate the transfer of the ADP-ribose moiety of NAD^+ to the α-subunit of G-proteins. In practice ^{32}P-labelled NAD^+ is used.
- Use of *N*-ethylmaleimide as a reversible inhibitor of α-subunits, which it selectively alkylates.
- Use of photoaffinity labelling using analogues of GTP such as GTP-azidoanilide, which is converted to a nitrene by light and covalently labels the α-subunit.
- Use of antibodies raised to each subunit.
- Use of gene cloning and site-directed mutagenesis techniques and expression of the clones in *Xenopus* oocytes.
- Production of chimera receptors by genetic engineering techniques.
- Synthesis and use of a range of ligand analogues to identify the structural and kinetic features of receptor–ligand binding. The advent of combinatorial chemistry, which allows the simultaneous synthesis of a very large number of structurally related compounds, has increased the importance of this approach.

The G-protein cycle

Agonist binding to the receptor triggers a G-protein cycle (Fig. 16.11):

- In the normal resting state, the trimeric G-protein has a molecule of GDP bound to the α-subunit. At this stage the G-protein is not coupled to the receptor but is nearby.
- Agonist binds to the receptor to form the active receptor–agonist complex, concomitantly inducing a rapid conformational change in the TM6 helix of the receptor that activates the G-protein-binding site located in intracellular loops. The complex interacts by diffusion translocation with a G-protein–adaptor complex and binds to the α-subunit. Numerically there are more G-proteins than receptor proteins in the membrane.
- Binding of the receptor–agonist complex to the G-protein–adaptor complex induces a conformational change in the guanine nucleotide-binding site on the α-subunit, causing the dissociation of the GDP and the formation of a transient 'empty state'.
- GTP binding to the nucleotide-binding site on the α-subunit triggers a rapid conformational change in the α-subunit and subsequent dissociation of the GTPα subunit, leaving the Gβγ-subunits as a dimer. Both the GTPα-subunit and the Gβγ-dimer remain attached to the cell membrane.

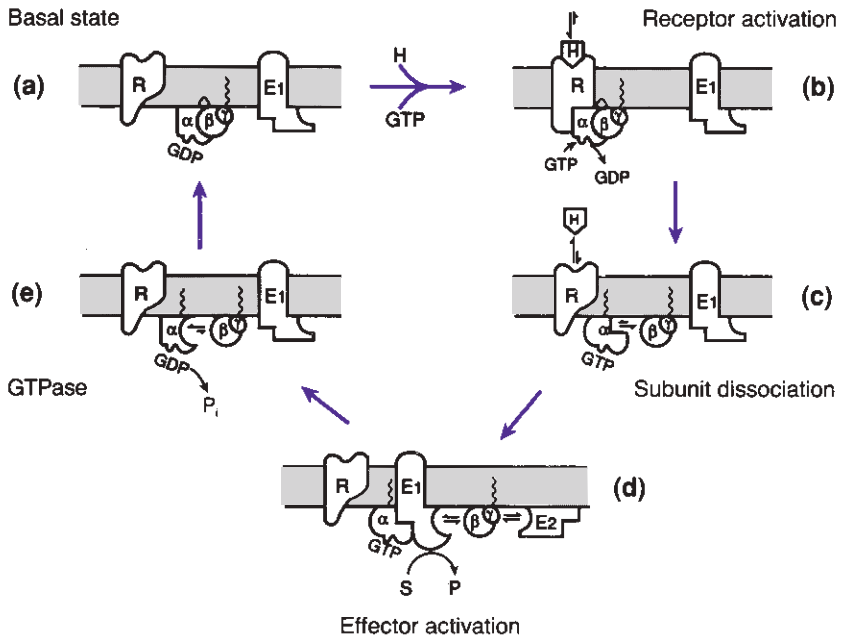


Fig. 16.11. G-protein-mediated transmembrane signal transduction. (a) The basal state consists of GDP tightly bound to the α-subunit of the heterotrimer. The receptor (R) is unoccupied and the effector (E_1), e.g. adenylyl cyclase, is inactive. (b) Ligand binding induces a conformational change, resulting in the replacement of GDP by GTP. (c) GTP binding causes the G-protein to dissociate from the receptor and the α-subunit–GTP complex (GTP_α) to dissociate from the βγ-subunits. (d) The GTP_α complex binds to, and activates, the effector. The βγ-subunit complex may activate a second effector (E_2). (e) The GTPase activity of the α-subunit causes the hydrolysis of GTP to GDP and the deactivation of, and dissociation from, the effector. The GDP_α reassociates with the βγ-complex, returning the system to its basal state. (Reproduced from J. R. Hepler and A. G. Gilman (1992), G-Proteins. *Trends in Biochemical Sciences*, **17**, 383–389, by permission of Elsevier Science.)

- The GTPα-subunit and/or the Gβγ-dimer binds to an inactive effector molecule causing its activation or inhibition.
- Hydrolysis of GTP to GDP by the GTPase site of the α-subunit terminates the activation or inhibition by reversing the conformational change originally induced by the receptor–agonist complex. This facilitates the dissociation of the α-subunit from the effector and its reassociation with the Gβγ-dimer, thus completing the cycle.

Each G-protein cycle results in a very large amplification of the original signal.

A given G-protein may be activated by a large number of different receptors (referred to as G-protein promiscuity), whilst a given receptor may interact with different G-proteins and/or produce more than one response (referred to as receptor promiscuity). A receptor capable of activating more than one type of G-protein and hence of initiating more than one response is referred to as a pleiotropic

receptor (meaning it has multiple phenotypic expressions). An example is the human calcitonin receptor, which can couple to G_i, G_s and G_q.

G-protein–effector coupling

It was originally believed that only the GTPα-subunit could interact with an effector but more recent research has shown that the Gβγ-dimer can also act as an independent transducer. Two important examples of the role of Gα-GTP as a transducer are the activation of adenylyl cyclase that converts ATP to the second messenger cAMP, and phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2), a component of the cytoplasmic side of the cell membrane, to two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. Most examples of the transducer role of the Gβγ-dimer are linked to the activation of G_i and G_0. Examples include the activation of β-adrenergic receptor kinase (βARK), phospholipase A_2 and the K^+ channel GIRK (*G*-protein-activated *i*nwardly *r*ectifying potassium channel).

Regulation of G-protein transduction

The heterogeneity of G-proteins and the diversity of the responses they may induce via the transduction pathways offer a number of biochemical advantages, including amplification, control and specificity. Each Gα-GTP complex on binding, for example to adenylyl cyclase, may result in the synthesis or release of many molecules of second messenger, each of which may induce multiple responses (amplification) from the other components of the cascade system to which they are linked. Control is exerted at the level of the receptor by virtue of the fact that binding of the Gα-GTP complex to the receptor results in a decreased affinity by the receptor for the ligand (increased K_d), thus encouraging the release of the ligand, whilst hydrolysis of GTP to GDP reverses the affinity change. Control is also exerted by a group of over 20 proteins collectively called RGS (*r*egulation of *G*-protein *s*ignalling) proteins that can be grouped into five subfamilies based on sequence homology. They all have the ability to bind to the Gα-GTP subunit at an RGS binding domain (Fig. 16.12), where they have two actions:

- they reduce the binding of the Gα-GTP to the effector,
- they act as GTPases, accelerating the hydrolysis of GTP to GDP by a factor of over 2000-fold.

Both actions result in the deactivation of the effector. This may allow the termination of the signal following removal of the stimulus or a redirection of a signal within a signalling network. There is evidence that specific RGS proteins regulate specific G-protein-coupled pathways. This specificity is determined by a combination of factors, including cell type-specific expression of RGS proteins, the intracellular localisation of the RGS proteins, the presence of domains other than the RGS domain that facilitate coupling to specific signalling pathways and the ability of some RGS proteins to act as effector antagonists that prevent a G-protein from coupling with its effector.

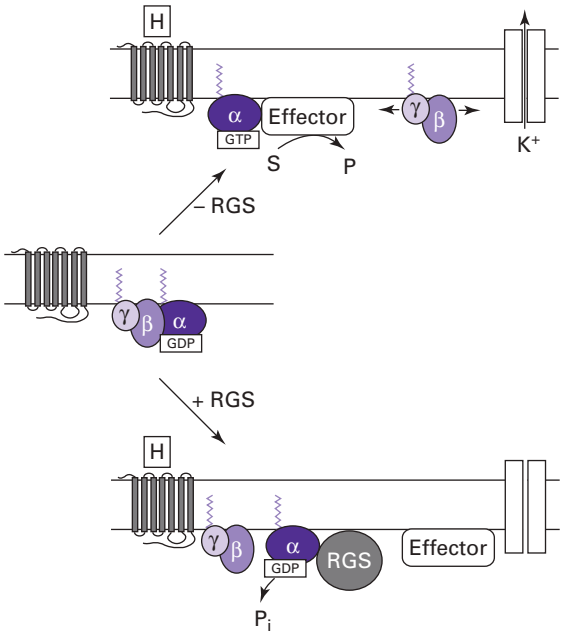


Fig. 16.12. Regulators of G-protein signalling (RGS) proteins negatively regulate receptor-directed G-protein signalling. In the resting state (centre), cell surface receptors for neurotransmitters and hormones are unoccupied by agonist, and G-proteins exist as a Gαβγ heterotrimer with GDP bound to the α-subunit. Hormone agonists (H) binding to the receptor (top) initiate guanosine nucleotide exchange, which facilitates GTP binding to the Gα-subunit and the release of the Gβγ. The active Gα-subunit–GTP complex and the Gβγ-dimer are free to regulate the activity of target effector proteins such as certain ion channels or enzymes that convert substrate molecules (S) into second-messenger products (P). Signalling is terminated as a result of the intrinsic GTPase activity of the Gα-subunit. Most of the RGS proteins bind to the active Gα-subunit–GTP complex (bottom) and greatly accelerate intrinsic Gα-subunit–GTPase activity. GTP hydrolysis by the Gα-subunit terminates Gα-subunit–effector interactions and promotes reassociation of the Gα-subunit–GDP complex with the Gβγ-dimer, which blocks Gβγ-dimer signalling. (Reproduced from J. R. Hepler (1999), Emerging roles for RGS proteins in cell signalling. *Trends in Pharmacological Sciences*, **20**, 376–382, by permission of Elsevier Science.)

Recent evidence indicates that phosphorylation may be an important activation mechanism for GPCRs. It has been established for some years that phosphorylation is important in receptor desensitisation (Section 16.6.1), and endocytosis (Section 16.6.2) but it appears that it may also be involved in coupling receptors to specific signalling pathways. Research with the muscarinic M_3 receptor, for example, has shown that it is phosphorylated by the enzyme casein kinase 1α at a site distinct from that leading to desensitisation and that this phosphorylation is essential for the activation of the extracellular-signal-regulated kinase 1 and 2 pathway (ERK-1 and 2).

16.5.3 Signal transduction through receptors with intrinsic protein kinase activity

It has been appreciated for over 20 years that phosphorylation coupled with dephosphorylation represents an important mechanism for the regulation of protein activity. A large number of intracellular kinases and phosphatases have been characterised and their regulatory action linked to conformational changes induced in the target protein as a result of the introduction or removal of a phosphate group. Phosphorylation can be studied by the use of ^{32}P-ATP. Control of protein activity by the kinase/phosphatase principle is found in a broad range of organisms, indicating its early evolution. It operates with the net consumption of ATP, but with the considerable gain in sensitivity, amplification and flexibility that more than compensate for the ATP consumed (for further details see Section 15.4).

More recently it has been shown that a large number of cell membrane receptors possess latent protein kinase activity that is activated by agonist binding. The transduction action of these receptors is linked to this kinase activity. Agonist binding induces the autophosphorylation of one or more tyrosine, serine or threonine residues within the intracellular region of the receptor protein and in the concomitant activation of the protein kinase activity of the intracellular domain of the receptor towards intracellular proteins. There are two main types of receptor kinase based on the specificity of the kinase.

Receptor tyrosine kinases

Twenty subclasses of receptor tyrosine kinases (RTKs) are known on the basis of their extra- and intracellular structures. Three of the best characterised are the EGF receptor, the insulin receptor and the PDGF receptor shown in Fig. 16.13. The majority of RTKs are single-chain, monomeric proteins in the absence of their agonist but dimerise on agonist binding. However, a few, including the insulin receptor, are dimeric but in all cases there is a single membrane-spanning domain within each monomer. There is evidence that, like G-protein-coupled receptors, the protein kinases interact with membrane-anchoring proteins of the Shank family type.

The agonist-binding site of the RTKs is located on the glycosylated extracellular region, whilst immediately after the transmembrane region there is the tyrosine kinase domain. Agonist binding causes a conformational change that allows the occupied receptors to recognise each other, resulting in their dimerisation. This is rapidly followed by the mutual cross-autophosphorylation of one to three tyrosine residues in the tyrosine kinase domains on each monomer, thus switching on their activity. The activated sites bind effectors, causing their phosphorylation and activation and the concomitant formation of receptor–signalling complexes. In this respect, therefore, these receptors resemble the G-protein-coupled receptors. These complexes are also stabilised by adaptor proteins but their identity is different from those associated with GPCRs. Examples include Gab-1, p85 and Grb2. The effectors and adaptors possess common binding domains for the tyrosine residues that have been phosphorylated by the kinase activities that may or may not be near

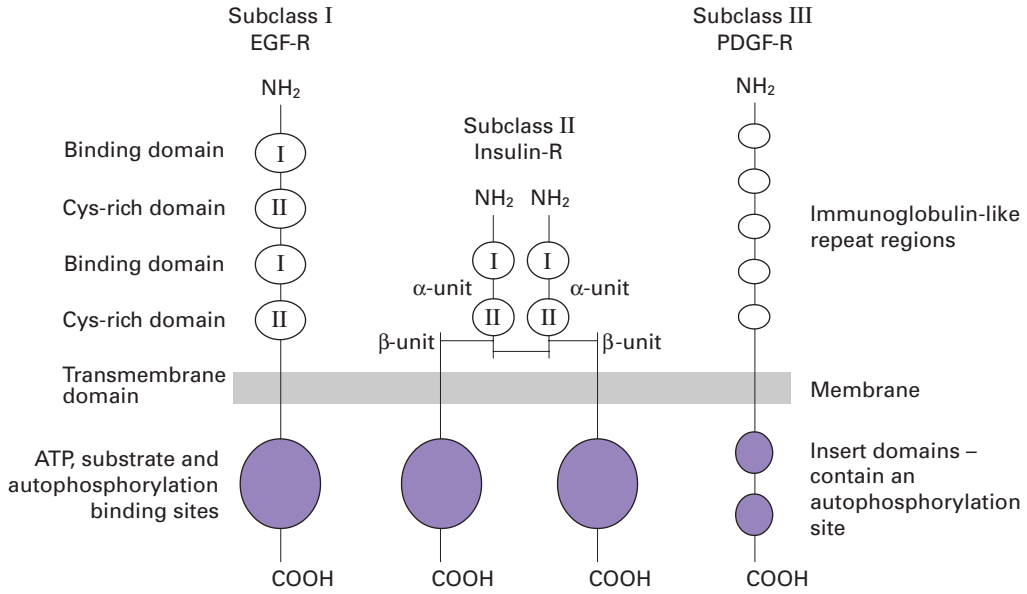


Fig. 16.13. Diagrammatic representation of three receptor tyrosine kinase subclasses. The EGF subclass contains two ligand-binding domains that are located in juxtaposition so that the ligand binds in a cleft between the two domains. The two cysteine-rich domains are both located near the membrane surface. On ligand binding, both the EGF and PDGF subclass receptors dimerise so that the intracellular tyrosine kinase domain possesses elevated activity and enhanced binding affinity relative to the monomeric forms. The insulin subclass receptors are effectively dimeric, but, as with the subclasses I and III, there is allosteric interaction between the two αβ halves of each receptor on ligand binding. The tyrosine kinase domains of the three subclasses show the greatest degree of homology between the three subclasses.

to the kinase active sites, including SH2 domains and phosphotyrosine-binding (PTB) domains. The effector may possess additional binding domains to which other effectors can bind. As a consequence of these additional domains the first effector is acting as an adaptor for the second effector, thereby enabling a web of signalling pathways with the potential for pathway branching to be established to meet prevailing cellular demands.

Receptor serine and threonine kinases

Complementary to the tyrosine kinase group of receptors is a second group of protein kinase receptors characterised by their ability to autophosphorylate serine and threonine residues in the intracellular domain of the receptor. These protein serine/threonine kinase receptors are specific for members of the transforming growth factor (TGF)-β superfamily that regulate growth, differentiation, migration and cell adhesion. They are classified into a number of subgroups on the basis of their structure, particularly their serine/threonine kinase domain. They are all single transmembrane receptors that, on binding of their ligand, form

hetero-oligomeric complexes between subgroup types. This stimulates autophosphorylation and activation of the serine/threonine kinase activity towards other cytosolic proteins that are components of the transduction pathway. Some growth factor receptors lack kinase activity after dimerisation and autophosphorylation. In these cases they bind intracellular kinases and operate as a normal receptor kinase.

Like the GPCRs, receptor protein kinases stimulate numerous transduction pathways. The downstream members of these transduction pathways include the phospholipases and phosphoinositide kinases that are also involved in the G-protein transduction pathways. Among a number of effectors unique to the protein kinases is Ras, a membrane-bound guanosine-binding protein with intrinsic GTPase activity that is involved in cell growth and development in all eukaryotes.

Crucial to the control of transduction by receptor kinases is the existence of a group of protein phosphatases that can either deactivate or activate pathways by dephosphorylation. Phosphatases specific for tyrosine and others that act on serine and threonine as well as tyrosine have been identified. Some are purely cytoplasmic whilst others are receptor-like with a transmembrane domain. Most have two phosphatase domains, for reasons that are not clear, but their specificity may be linked to interaction between the two sites. The activity of the phosphatases appears to be linked to their own phosphorylation and a significant number have an SH2 domain for the receptor tyrosine kinases.

16.6 RECEPTOR DESENSITISATION AND TRAFFICKING

16.6.1 Receptor desensitisation

One of the characteristics of all of the three major groups of cell membrane receptors is that they display desensitisation characterised by a reduction or termination of the cellular response despite the continued presence of the agonist. However, from a kinetic perspective there are differences in the rate of onset of their desensitisation between the three classes. Thus, with ligand-gated ion channels, typified by the nAChR, desensitisation occurs within seconds of agonist activation whereas with GPCRs and protein kinase receptors desensitisation takes minutes and occasionally hours to occur. Such differences are a clear indication that different mechanisms are responsible for this loss of activity. In the case of the nAChR, the desensitised conformation of the receptor has a higher affinity for the agonist than has the active open channel conformation and this higher affinity alone is sufficient to drive the transition to the desensitised state but not before the ion channel has opened and closed many times. Removal of the agonist immediately reverses the desensitisation. It is known that some ligand-gated ion channels also undergo phosphorylation following activation and this too may facilitate desensitisation but by a kinetically slower route.

The kinetically slow onset of desensitisation of GPCRs and protein kinase receptors is believed to be linked to covalent modification of the receptor by phosphorylation at a site that is distinct from that of the autophosphorylation of the receptor

that is an essential part of their activation. Postactivation phosphorylation of a tyrosine, serine or threonine residue in the intracellular region of the receptor introduces a polar phosphate group that can induce conformational changes deleterious to the normal functioning of the receptor. Studies with GPCRs have shown that phosphorylation by the second-messenger-activated kinases such as cAMP-dependent kinase and protein kinase A results in the uncoupling of the receptor from its G-protein. Phosphorylation can also be promoted by a family of seven serine/threonine GPCRs (GRK-1–7) that are capable of acting on agonist-occupied or constitutively active GPCRs. Phosphorylation of receptors has also been shown to trigger the internalisation (sequestration) and trafficking (recycling and degradation) of receptors. Internalisation may occur by a number of mechanisms, the most thoroughly understood of which is endocytosis.

16.6.2 Endocytosis and receptor trafficking

The internalisation and trafficking of receptors has been most thoroughly investigated using GPCRs, particularly the β_2-adrenergic receptor. Studies have revealed that the processes involve three stages:

- The recruitment of the receptors, normally with agonist bound to its binding site (agonist-dependent internalisation), but in some cases in the absence of bound agonist (agonist-independent internalisation), to discrete endocytic sites in the membrane. In the case of the agonist-dependent route, the receptor is phosphorylated by a GRK prior to internalisation.
- The internalisation of the receptors to form an early endosome or receptosome.
- The intracellular sorting of the endosome for either the subsequent recycling of the receptors or for their degradation following the fusion of the endosome with a lysosome.

Endocytosis is the process by which extracellular molecules and membrane proteins, including receptors, are taken up into the cell. The uptake process for receptors has been investigated using the fluorescence technique of tagging the receptor with green fluorescent protein or one of its several genetically modified variants (Section 16.3.1). In the case of G-protein-coupled receptors, after binding the ligand and G-protein, the receptors undergo ligand-dependent phosphorylation by a GRK and this stimulates interaction with one of the β-arrestin family of protein adaptor complexes. These cytosolic adaptors facilitate the disruption of the interaction of the receptors with G-proteins and the recruitment of the receptors into coated pits in the membrane where they link the receptor to a protein called clathrin via a second adaptor protein called AP-2. This protein interacts with phosphoinositides and promotes both the assembly of the coated pits and the recruitment of the activated receptors to them. A Tyr-X-Arg-Phe-region near the C-terminal region of the receptor binds to one end of AP-2, whilst the other end binds to the clathrin. Coated pits are regions in the membrane that are rich in clathrin, which is located on the cytoplasmic side of the membrane. The pits tend to accumulate in one area of the cell, a process known as patching, and eventually

coalesce. Clathrin consists of three heavy chains and the light chains that can polymerise to form a polymeric cage-like structure or lattice that links to the C-terminal end of the receptor. The polymerisation of clathrin into the network involves the phosphorylation of the light chains at several sites, possibly involving different kinases. This phosphorylation is energy dependent and drives the whole endocytosis process. Ca^{2+} are involved in the stabilisation of the clathrin network. The polymeric clathrin network drives membrane deformation and the 'budding' of the coated pit to create an endocytic vesicle. Once the vesicle has formed, the clathrin coat is lost as a result of the action of one of the proteins of the heat shock protein family (hsp 70). The vesicles then fuse to form an early endosome or receptosome (Fig. 16.14). The formation of endosomes leaves the cytoplasmic region of the receptor exposed to the external environment. A number of adaptor molecules in addition to AP-1 and AP-2 have been identified and there is evidence that two of them, AP-3 and AP-4, may act independently of clathrin. The molecular details of the agonist-independent route of receptor internalisation are less well established. It appears that arrestins and GRKs are not required but that AP-2 and clathrin-coated pits are essential for the formation of an endosome.

Once the endosome is within the cell, the clathrin coat is depolymerised probably by hsp 70, with the concomitant hydrolysis of ATP. There are two possible fates for the receptors within the endosome:

- dephosphorylation followed by recycling to the membrane, thereby restoring a functional receptor;
- proteolysis resulting a net decrease in membrane receptor function, a process known as downregulation.

The pathways for endocytic sorting are determined by the operation of sorting signals. The main sorting signal appears to reside in the cytoplasmic region of the receptor itself. Thus, for the β_2-adrenergic receptor, a PDZ-binding domain in the C-terminal region interacts with a protein called ezrin–moesin-binding phosphoprotein 50 (EBP50), with the result that the receptor undergoes recycling, the process also involving arrestin and a protein known as *N*-ethylmaleimide-sensitive fusion protein (NSF) (Fig. 16.14). Chimeras of the receptor lacking the PDZ domain are directed to the degradative pathway. Receptors sorted for recycling are first dephosphorylated by an endosome-associated phosphatase and recycled back to the membrane via the Golgi complex. In the case of receptors directed to degradation the sorting signal again appears to reside in its C-terminal region. In these cases this region of the receptor interacts with a sorting protein called nexin 1, which promotes the fusion of the endosome with a lysosome, the resulting decrease in pH to 5.3 within the vesicle facilitating the downregulation of the receptor by proteolysis. There is evidence that this downregulation process is also dependent upon the ubiquitination of the receptor, a process that may include an active role for arrestin. Ubiquitin is known to 'tag' proteins for degradation, the process involving the action of a number of proteasomes (Section 15.5.4).

Much of the research on the mechanism of receptor endocytosis has been carried out using relatively few GPCRs and a few receptor protein kinases such as the

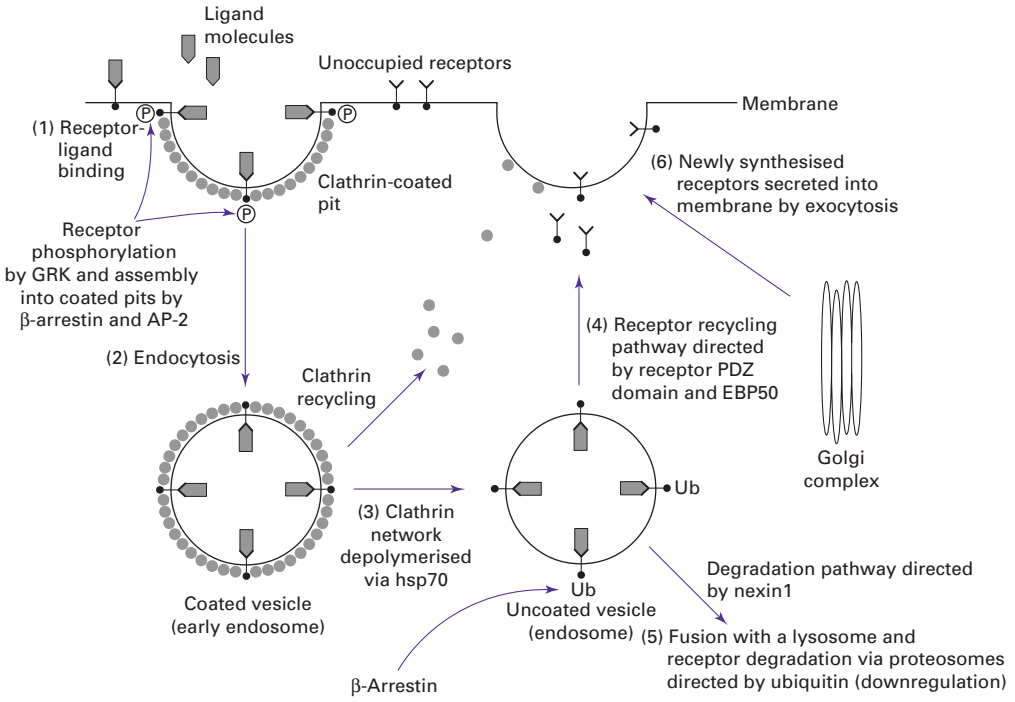


Fig. 16.14. Pathway of agonist-dependent G-protein-coupled receptor internalisation and endocytic sorting. (1) Occupied receptors are phosphorylated (Ⓟ) by a G-protein-coupled receptor kinase (GRK), leading to the recruitment of arrestins. Arrestins serve as adaptor proteins by linking phosphorylated receptors to components of the transport machinery such as clathrin and adaptor protein AP-2 and their recruitment to clathrin-coated pits. (2) The coated pit 'buds' into the cytoplasm aided by the clathrin, which forms a network, leading to the formation of an endosome. Note that the cytoplasmic domain of the receptor remains exposed to the cytoplasm following endocytosis. (3) The clathrin network is depolymerised and the clathrin recycled to the inner membrane. (4) The receptors are dephosphorylated and as a result of the interaction of EBP50 with a PDZ domain on the receptor, traffic back to the cell surface, resulting in functional resensitisation. Alternatively, (5) dephosphorylated receptors are tagged with ubiquitin (Ub) and enter the degradation pathway. Here they interact with nexin1, which promotes the fusion of the endosome with a lysosome and the degradation of the receptor by a number of proteasomes – a process known as downregulation. (6) The Golgi complex secretes newly synthesised receptor molecules to the outer membrane surface by exocytosis. The balance between receptor cycling, receptor degradation and receptor synthesis, and exocytosis determines the number of functionally active receptors on the membrane surface at any time.

epidermal growth factor receptor (EGR). It has yet to be established just how universal the clathrin-linked endocytotic pathway is among the large number of other receptors and various cell types. What is very clear is that the expression, regulation and desensitisation of receptors are dependent on numerous protein–protein interactions, many of which occur at the plasma membrane interface, that each cause crucial conformational changes in the receptor, and/or their regulators such

as to couple receptor activity to current cellular and whole organism demands. It is equally evident that reversible multisite phosphorylation plays a vital role in the regulation of the activity of receptors and their effectors.

The temporal variation in the number of cell surface receptors available for ligand binding is the net result of receptor trafficking and of new receptor synthesis, which takes place in the rough endoplasmic reticulum. A leader sequence in the protein results in its recognition and transport to the Golgi complex where it is glycosylated, packaged into coated vesicles and inserted into the membrane by exocytosis, in which clathrin plays a vital role. The balance between receptor synthesis, recycling and degradation is subject to various control mechanisms so that free receptor availability in the outer membrane meets current physiological needs. Temporal variations in cell membrane receptor numbers are also of significance in the clinical response to chronic drug administration that leads to the downregulation of receptor numbers, and also in neurodegenerative conditions in which the release of the physiological agonist is deficient, resulting in upregulation of receptor numbers.

16.7 SUGGESTIONS FOR FURTHER READING

General

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HARDING, S. E. and CHOUDHRY, B. Z. (ed.) (2001). *Protein–Ligand Interactions: Structure and Spectroscopy*, Oxford University Press, Oxford. (Particularly valuable for the coverage of the applications of various spectroscopic techniques to the study of receptor structure and ligand binding.)

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Useful websites

G-proteins

<www.gpcr.org>
<www.cis.upenn.edu/~krice/receptor.html>

Receptor endocytosis

<www.cytochemistry.net/cell-biology/recend.htm>
<cellbio.utmb.edu/cellbio/recend.htm>

Techniques

<www.biochem.arizona.edu/tollin/cpwr/principl.htm>
<www.biacore.com>