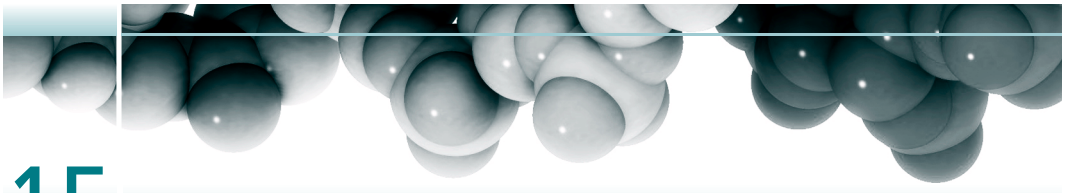


15 Enzymes

K. WILSON

15.1 CHARACTERISTICS AND NOMENCLATURE

15.1.1 Specificity and nomenclature

Enzymes are nature's biological catalysts possessing the ability to promote specific chemical reactions under the mild conditions that prevail in most living organisms. They are all proteins but range widely in their size from as few as 60–70 amino acid residues as in RNase to as many as several thousand. Generally they are much larger than their substrates and bind with them by means of **active sites** created by the specific three-dimensional folding of the protein. Interaction of specific functional groups in a small number of amino acid residues lining the active site with the substrate results in the formation of a **transition state** for which the activation energy barrier is significantly reduced relative to the non-enzyme-catalysed reaction. As a result, the reaction rate is increased by a factor of many millions relative to the uncatalysed reaction. Enzymes do not alter the position of equilibrium of reversible reactions that they catalyse but they do accelerate the establishment of the position of equilibrium for the reaction.

Many enzymes are members of coordinated metabolic or signalling pathways that collectively are responsible for maintaining a cell's metabolic needs under varying physiological conditions (Sections 15.5 and 17.4.5). The over- or under-expression of an enzyme can lead to cell dysfunction which we may recognise as a particular disease state. Enzyme inhibitors are widely used as therapeutic agents for the treatment of such conditions (Sections 15.2 and 18.1). Organ damage, for example heart muscle as a result of deprivation of oxygen following a heart attack, or the liver as a result of chemical damage as in alcoholic cirrhosis, results in the release of cellular enzymes into extracellular fluids and eventually into the blood. Such release can be clinically

monitored to aid diagnosis of the organ damage and to make a prognosis for the patient's future recovery (Section 16.3).

Enzymes are believed to catalyse over 4 000 different reactions but individual enzymes are characterised by their **specificity** for a particular type of chemical reaction. As a generalisation, enzymes involved in biosynthetic or signalling reactions show a higher specificity than ones involved in degradation reactions. **Bond specificity** is characteristic of enzymes such as peptidases and esterases that hydrolyse specific bond types. The specificity of these enzymes is determined by the presence of specific functional groups within the substrate 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, see Section 17.4.4) or sugar (e.g. hexokinase). DNA polymerase has a high specificity not only copying the base sequence of the DNA but also checking the product for accuracy afterwards. Enzymes may also display **stereospecificity** and be able to distinguish between optical and geometrical isomers of substrates. Enzymes have a **high capacity for regulation** in that the activity of enzymes that control the rate of a particular metabolic or signalling pathway can be enhanced or reduced in response to changing intracellular and extracellular demands. A range of regulatory mechanisms operates to allow short, medium and long-term changes in activity (Section 15.5.2).

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. This 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 and carbamyltransferases.
- *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 an 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 which 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 identify fully all the reactants involved. For example, glyceraldehyde-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.

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+}. DNA and RNA polymerases and many nucleases, for example, require two divalent cations for their active site. The cations correctly orientate the substrate and promote acid–base catalysis.

15.1.2 Isoenzymes and multienzyme complexes

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) which exists in five isoforms. LD is a tetramer which 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 16.3).

Multienzyme complexes

Some enzymes that promote consecutive reactions in a metabolic pathway associate to form a multienzyme complex. Examples include the 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 15.5.4).

Units of enzyme activity

Units of enzyme activity are expressed either in the SI units of **katals** (defined as the number of moles of substrate consumed or product formed per second) or **international units** (number of μmoles of substrate consumed or product formed per minute). Allied to activity units is **specific activity** which expresses the number of international units 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 ENZYME STEADY-STATE KINETICS

15.2.1 Monomeric enzymes

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 Section 15.3.3. 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**, ν_0, 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 many enzymes, the initial rate, ν_0, varies hyperbolically with substrate concentration for a fixed concentration of enzyme (Fig. 15.3). The mathematical equation expressing this hyperbolic relationship between initial rate and substrate concentration is known as the **Michaelis–Menten equation**:

$$\nu_0 = \frac{V_{max}[S]}{K_m + [S]} \qquad (15.1)$$

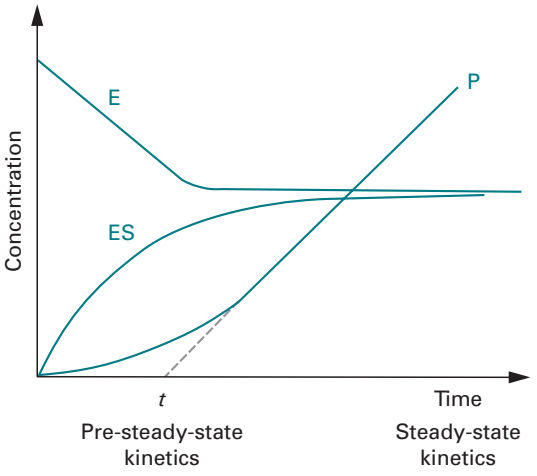


Fig. 15.1 Pre-steady-state progress curve for the interaction of an enzyme (E) with its substrate (S). P, product; *t*, induction time.

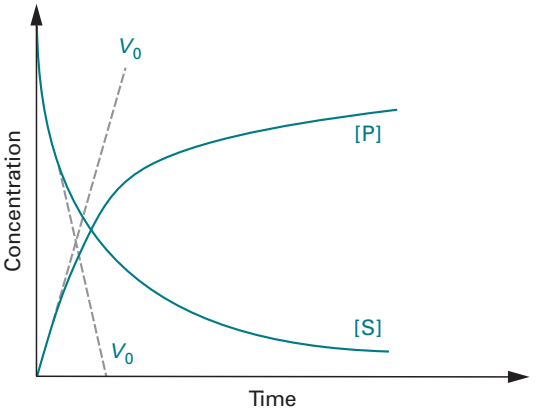


Fig. 15.2 Calculation of initial rate (ν_0) from the time-dependent change in the concentration of substrate (S) and product (P) of an enzyme-catalysed reaction.

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 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 can be formed and **zero-order** or **saturation kinetics** are observed. Under these conditions the reaction rate is only dependent upon the conversion of the enzyme–substrate complex, ES, to products and the diffusion of the products from the enzyme.

It can be seen from equation 15.1 that when $\nu_0 = 0.5\, V_{max}$, $K_m = [S]$. Thus K_m is numerically equal to the substrate concentration at which the initial rate is one-half of

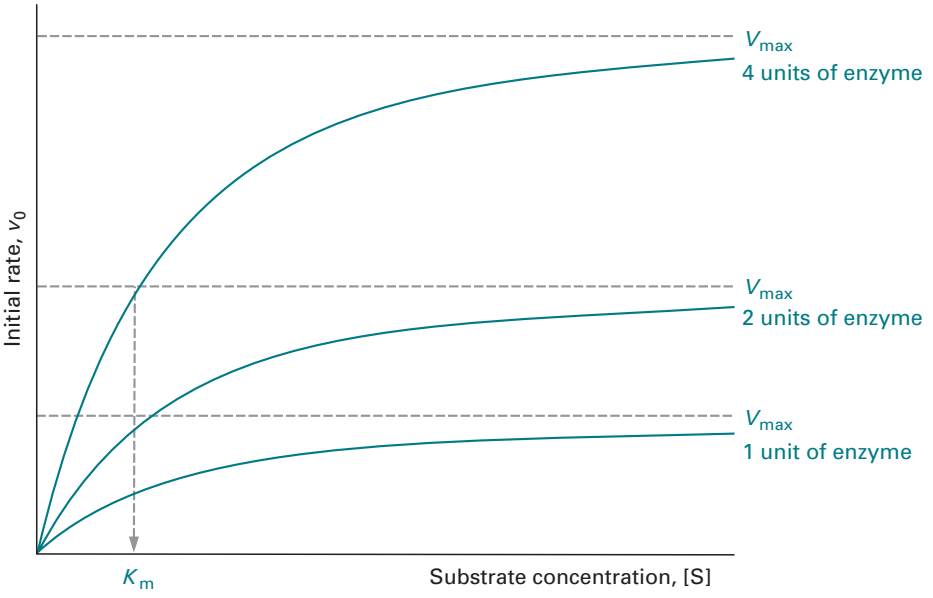


Fig. 15.3 The effect of substrate concentration on the initial rate of an enzyme-catalysed reaction in the presence of three different concentrations of enzyme. Doubling the enzyme concentration doubles the maximum initial rate, V_{max}, but has no effect on K_m.

the maximum rate (Fig. 15.3) 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] » K_m, equation 15.1 reduces to $\nu_0 \approx V_{max}$, but a simple calculation reveals that when $[S] = 10\,V_{max}$, ν_0 is only 90% V_{max} and that when $[S] = 100\,K_m$, $\nu_0 = 99\%\,V_{max}$. Appreciation of this relationship is vital in enzyme assays.

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:

$$k_{+1}[E][S] = k_{-1}[ES] \qquad (15.2)$$

hence:

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

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:

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

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:

$$E + S \underset{}{\overset{k_{+2}}{\rightleftarrows}} ES \rightarrow P_1 + EA \overset{k_{+3}}{\rightarrow} E + P_2$$

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 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 commonly used alternatives. The most popular of these is the **Lineweaver–Burk equation** obtained by taking the reciprocal of the Michaelis–Menten equation:

$$\frac{1}{\nu_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \tag{15.5}$$

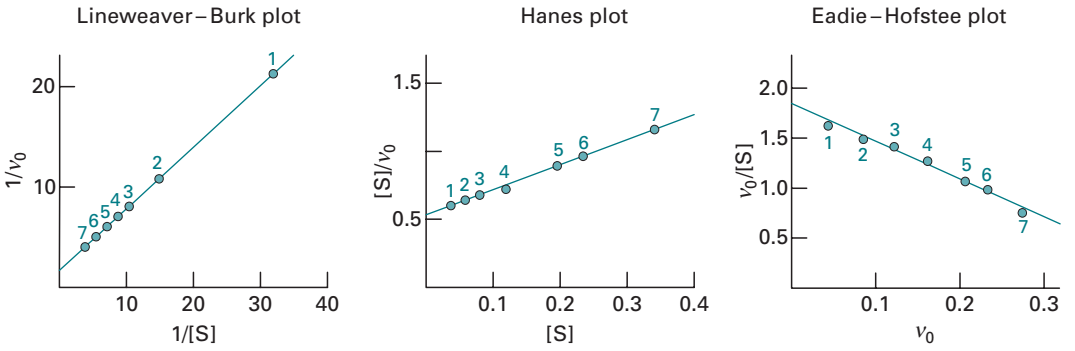


Fig. 15.4 Lineweaver–Burk, Hanes and Eadie–Hofstee plots for the same set of experimental data of the effect of substrate concentration on the initial rate of an enzyme-catalysed reaction.

A plot of $1/v_0$ against $1/[S]$ gives a straight line of slope K_m/V_{max}, with an intercept on the $1/\nu_0$ 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**:

$$\frac{[S]}{\nu_0} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \tag{15.6}$$

so that $[S]/\nu_0$ is plotted against $[S]$, and on the **Eadie–Hofstee equation**:

$$\frac{\nu_0}{[S]} = \frac{V_{max}}{K_m} - \frac{\nu_0}{K_m} \tag{15.7}$$

so that $\nu_0/[S]$ is plotted against ν_0. 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. 15.4 using the same set of experimental values of ν_0 for a series of substrate concentrations (for further details, see Example 1).

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. In spite of their widespread use, these linear transformations of enzyme kinetic data are subject to error. Specifically, they assume that the scatter of points around the line follows a Gaussian distribution and that the standard deviation of each point is the same. In practice this is rarely true. With the advent of widely available non-linear regression software packages such as DynaFit (www.biokin.com) and BRENDA (www.brenda-enzymes.info), there are now strong arguments for their preferential use in cases where accurate kinetic data are required.

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. 15.3 and is discussed further in Example 1. A valuable catalytic constant in addition to K_m and V_{max} is the **turnover number**, k_{cat}, defined as:

Example 1 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 (see Section 15.2.1 for further details). 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, and 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 these 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 these data are shown in Fig. 15.6 in which v_0 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 15.2.1, 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 are 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_0 values in units of μmol min^{-1} can be calculated. The data for the three linear plots are presented in Table B.

Example 1 (*cont.*)

Table B

[S] (mM)	0.03	0.06	0.09	0.12	0.18	0.24	0.36
v_0 (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
$1/v_0$ (μmol $min^{-1})^{-1}$	18.52	10.42	7.25	5.95	4.76	3.98	3.40
$v_0/[S] \times 10^3$ (dm^3 min^{-1})	1.8	1.6	1.53	1.40	1.17	1.05	0.82
$[S]/v_0 \times 10^{-3}$ (min dm^{-3})	0.56	0.63	0.65	0.71	0.85	0.95	1.22

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	−3.99	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 the value of 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 V_{max} of 51 μmol $min^{-1}dm^{-3}$, but it is only possible to correct for enzyme amount if it was pure and of known amount in molar terms. The enzyme is known to have a molecular mass of 68 kDa so if there was 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):

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

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 180 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:

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

Example 1 (*cont.*)

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 and is lower than the limiting value.

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

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 4×10^7 s^{-1} and is one of the most efficient enzymes known. The catalytic potential of high turnover numbers can only be realised 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 $M^{-1}\,s^{-1}$.

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 rate constant value of about 10^9 $M^{-1}\,s^{-1}$ 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 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 $M^{-1}\,s^{-1}$ with a K_m of 1.1 M (very high), whilst fumerase 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 as a consequence of its juxtaposition in the complex, thereby eliminating diffusion limitations (Section 15.4.2).

Effect of enzyme concentration

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

$$v_0 = \frac{k_{+2}[E][S]}{K_m + [S]}$$

and hence that

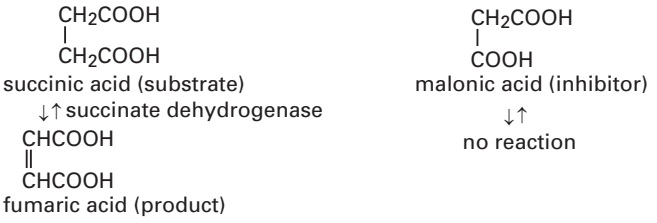
$$v_0 = \frac{k_{+2}[E]}{(K_m/[S]) + 1} \tag{15.9}$$

Thus, when the substrate concentration is very large, equation 15.9 reduces to $v_0 = k_{+2}$ [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 15.3). Figure 15.3 illustrates the importance of the correct measurement of initial rate.

15.2.2 Inhibition of monomeric enzyme reactions

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}). For competitive inhibition the following two equations can be written:

$$E + S \rightleftharpoons ES \longrightarrow E + P$$
$$E + I \rightleftharpoons EI \longrightarrow \text{no reaction}$$

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 $v_0 = 0.5V_{max}$ then:

$$[S] = K_m \left(1 + \frac{[I]}{K_i}\right) \tag{15.10}$$

where [I] is the concentration of inhibitor.

It can be seen from equation 15.10 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:

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]}\left(1 + \frac{[I]}{K_i}\right) + \frac{1}{V_{max}} \tag{15.11}$$

Application of this equation allows the diagnosis of competitive inhibition (Fig. 15.5a). 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

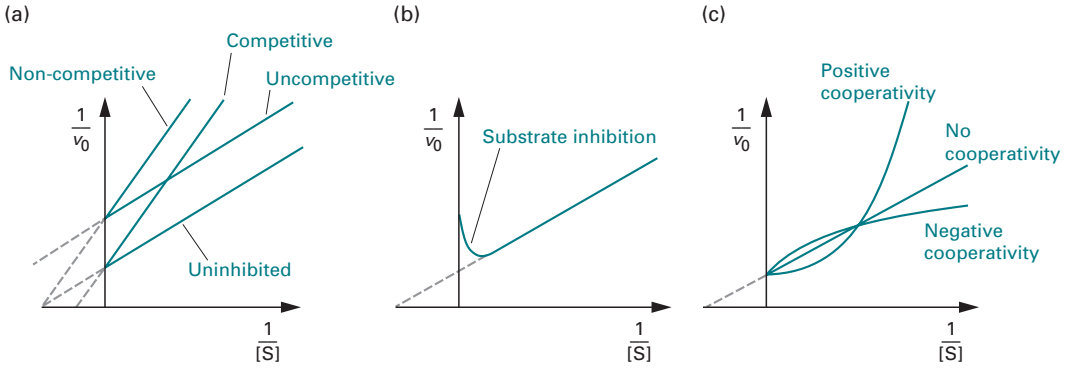


Fig. 15.5 Lineweaver–Burk plots showing (a) the effects of three types of reversible inhibitor, (b) substrate inhibition and (c) homotropic cooperativity.

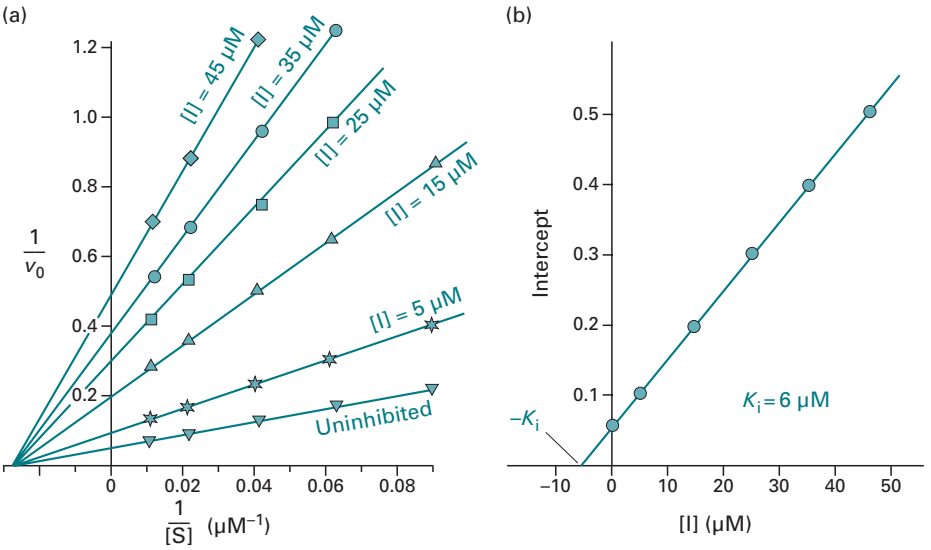


Fig. 15.6 (a) Primary Lineweaver–Burk plots showing the effect of a simple linear non-competitive inhibitor at a series of concentrations and (b) the corresponding secondary plot that enables the inhibitor constant K_i to be calculated.

value is obtained from a secondary plot (Fig. 15.6). 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:

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \tag{15.12}$$

Once non-competitive inhibition has been diagnosed (Fig. 15.5a), 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 $1/v_0$ axis) against inhibitor concentration. Both secondary plots will have an intercept of $-K_i$ on the inhibitor concentration axis (Fig. 15.6).

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.

$$\begin{array}{ccccc} E + S & \rightleftharpoons & ES & \longrightarrow & E + P \\ & & +I \downarrow\uparrow -I & & \\ & & ESI & \longrightarrow & \text{no reaction} \end{array}$$

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:

$$\frac{1}{v_0} = \left(\frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}\right)\left(1 + \frac{[I]}{K_i}\right) \tag{15.13}$$

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. 15.5a. 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. 15.5b. It is explicable in terms of the substrate acting as an uncompetitive inhibitor and forming a dead-end complex.

End-product inhibition

The 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 in the pathway thus switching off the whole pathway when the final product begins to accumulate. The inhibition of aspartate carbamyltransferase by cytosine triphosphate (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.

Irreversible inhibition

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 15.4.1).

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 15.4.1);

- it gives an understanding of the possible ways by which metabolic activity may be controlled *in vivo*;
- it allows specific inhibitors to be synthesised and used as therapeutic agents to block key metabolic pathways underlying clinical conditions (Section 18.1.2).

15.2.3 Effect of temperature and pH on enzyme reactions

Effect of temperature

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

$$\text{rate} = Ae^{-E/RT} \tag{15.14}$$

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 15.14 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 °C rise in temperature (**Q_{10} value**). At a temperature characteristic of the enzyme, and generally in the region 40 to 70 °C, the enzyme is denatured and enzyme activity is lost. The activity displayed in this 40 to 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 combination of thermal stability, temperature coefficient and incubation time 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 15.3). Interestingly, recent work with enzymes from mesophiles and thermophiles have 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 a **transition state**, which is a transient intermediate in the formation of the product(s) from the substrate(s), that has a lower energy barrier than that for the non-catalysed reaction. This results in a decrease in the activation energy (E_{act}) for the reaction relative to that for the non-enzyme-catalysed reaction (Fig. 15.7). A decrease in the energy barrier of as little as $5.7\,kJ\,mol^{-1}$, equivalent in energy terms to the strength of 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 occurs in the uncatalysed reaction! Figure 15.7 shows a simple energy profile for the conversion of

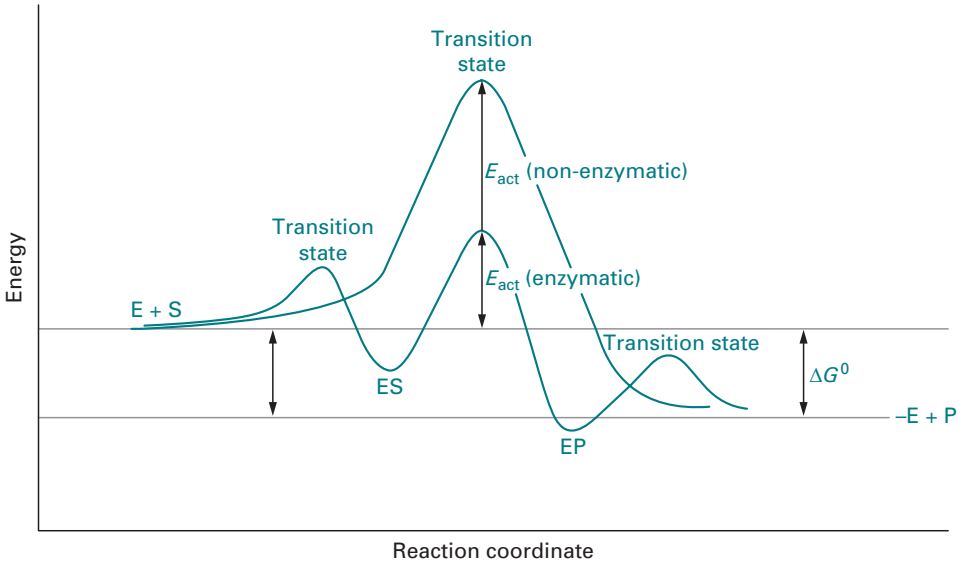


Fig. 15.7 Energy profile of a simple enzyme-catalysed reaction. The formation of ES and EP and the subsequent release of E + P proceeds via several transition states. The activation energy for the overall reaction is dictated by the initial free energy of E and S and the highest energy transition state. 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.

a substrate to product 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 overall reaction and hence its rate, is the formation of one or more intermediates in which covalent bonds are being made and broken and which cannot be isolated. 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).

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:

$$\Delta G^0 = -RT \ln K_a \tag{15.15}$$

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

$$\ln K_a = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT} \tag{15.16}$$

A small number of enzymes appear to operate by a mechanism that does not rely on the formation of a transition state. Studies with the enzyme methylamine dehydrogenase, which promotes the cleavage of a C–H bond, have shown that the reaction is independent of temperature and hence is inconsistent with transition state theory.

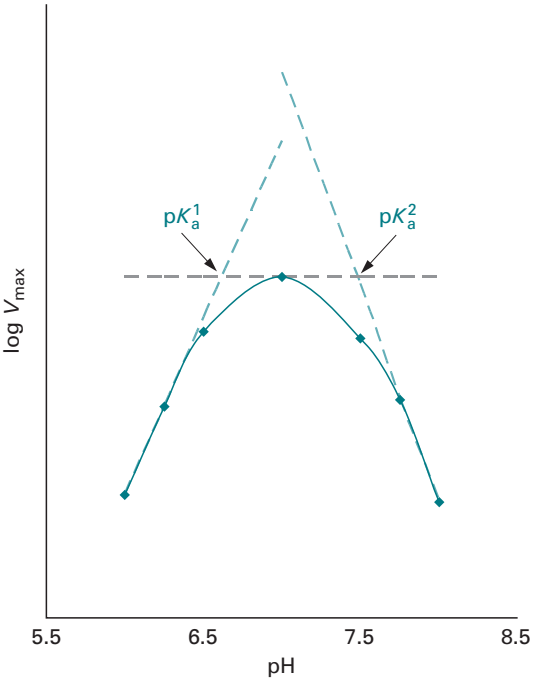


Fig. 15.8 The effect of pH on V_{max} of an enzyme-catalysed reaction involving two ionisable groups in the active site of the enzyme. The construction of tangents to the experimental line allows the pK_a values of the ionisable groups to be estimated.

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 the barrier (hence 'tunnelling') at an energy level near 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.

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 they 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. 15.8).

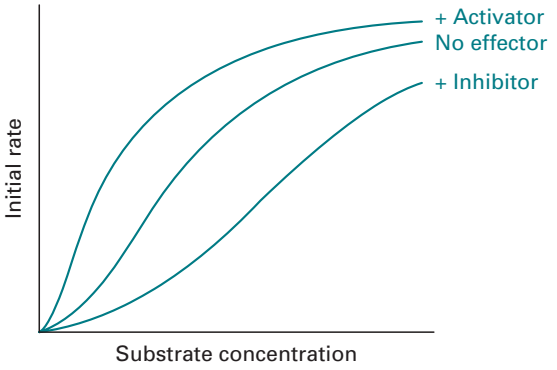


Fig. 15.9 Effect of activators and inhibitors on the sigmoidal kinetics of an enzyme subject to allosteric control.

15.2.4 Allosterism and cooperativity

The discussion of the mechanism of enzyme action so far has been based on the assumption that successive substrate molecules bind to the enzyme with the same ease (affinity). This is not true with some enzymes. With some enzymes successive binding occurs with either progressive greater ease or with reduced ease and the enzymes are said to be subject to **allosteric control.** Historically, all such enzymes were believed to possess quaternary structure and consist of several protein subunits (**oligomers**), which could be identical or different, and to possess multiple catalytic sites. Proteins subject to allosteric control are not confined to enzymes. Other examples are haemoglobin and many cell membrane receptors especially those of the G-protein-coupled receptors (GPCRs) type (Section 17.4.3). Oligomeric enzymes may either display simple Michaelis–Menten kinetics in which case all substrate molecules bind with equal ease, as is the case with the tetramer lactate dehydrogenase (Section 15.1.2), or they display a characteristic **sigmoidal** relationship between initial rate and substrate concentration (Fig. 15.9) indicative of an **allosteric enzyme**. Examples of such enzymes are aspartate carbamoylase and phosphofructokinase. Progressive binding of the substrate molecules to the subunits of an allosteric enzyme may result in either increased (**positive cooperativity**) or decreased (**negative cooperativity**) activity towards the binding of further substrate molecules. In such cases the substrate molecules are said to display a **homotropic effect**. Changes in catalytic activity towards the substrate may also be brought about 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. 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. 15.9). The diagnosis of cooperativity by use of the Lineweaver–Burk plot is shown in Fig. 15.5c. The operation of cooperative effects may be confirmed by a Hill plot, which is based on the equation:

$$\log \frac{v_0}{V_{max} - v_0} = h \log[S] + \log K \quad (15.17)$$

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.

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.

Two classical models have been proposed to interpret allosteric regulation. They are both 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. The first such model was due to Jacques Monod, Jeffries Wyman and Jean-Pierre Changeux, and is referred to as 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. 15.10). The second model of Daniel Koshland, known as the **induced-fit** or **sequential model**, 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 modifies the reactivity of others.

Recent research has shown that allosterism is not confined to oligomeric proteins since some monomeric proteins may also display the behaviour (Fig. 15.10). The emerging opinion is that allostery is a consequence of the flexibility of proteins such that the continuous folding and unfolding in localised regions of the protein gives rise to a population of conformations that interconvert on various timescales, which differ in their affinity for certain ligands and, in the case of enzymes, in their catalytic activity. The interconverting conformations have similar energies and their mixture constitutes the 'native state' of the protein. The binding of an allosteric effector at its distinct site results in the redistribution of the conformational ensembles as a result of the alteration of their rates of interconversion, and as a consequence, the conformation and hence activity of the active site is modified. Integral to this redistribution

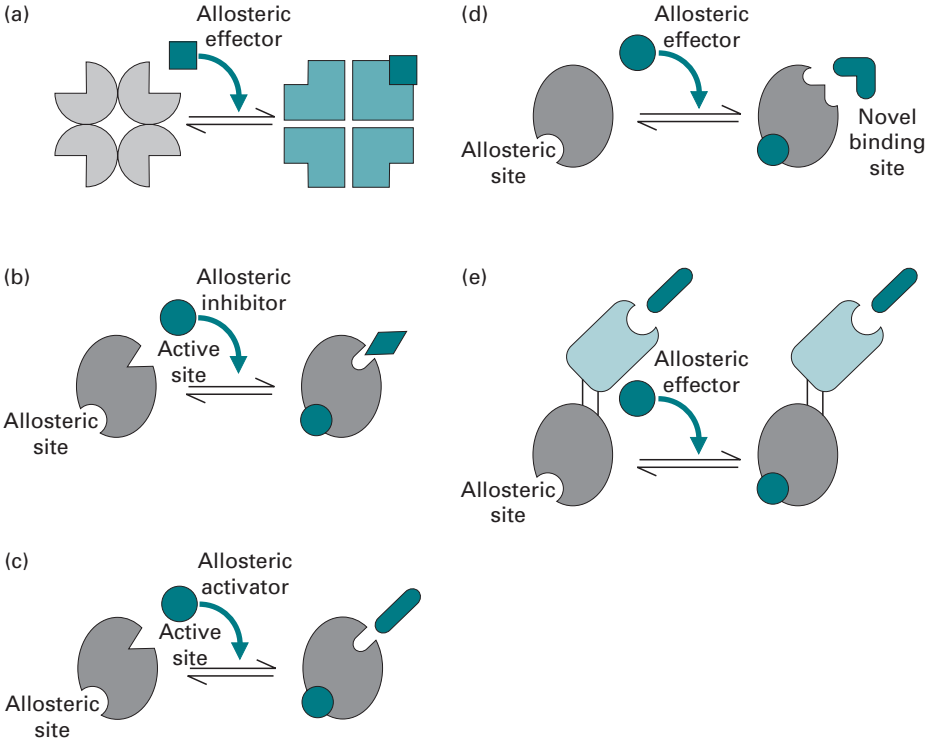


Fig. 15.10 Different modes of allosteric behaviour. (a) A representation of the Monod–Wyman–Changeux model of allosteric transitions. A symmetric, multimeric protein can exist in one of two distinct conformational states – the active and inactive conformations. Each subunit has a binding site for an allosteric effector as well as an active site or binding site. (b) A monomeric, allosterically inhibited protein. The binding of an allosteric inhibitor alters the active site or binding site geometry in an unfavourable way, thereby decreasing affinity or catalytic efficiency. (c) A monomeric, allosterically activated protein. The binding of an allosteric activator results in an increased affinity or activity in the second site. (d) The binding of an allosteric effector might introduce a new binding site to a protein. Binding of a ligand to this new binding site could lead to changes in active site geometry, providing an indirect mechanism of allosteric control. This type of effect is of great interest in the design of allosteric drugs and can be considered as a subset of the example in (c). (e) The fusion of an enzyme to a protein under allosteric control. This type of construct can act as an allosteric switch because the activity of the enzyme is indirectly under allosteric control via the bound protein with an allosteric site. Such constructs are both present in nature and the target of protein engineering studies. (Reproduced from Nina M. Goodey and Stephen J. Benkovic (2008). Allosteric regulation and catalysis emerge via a common route. *Nature Chemical Biology*, **4**, 474–482, by permission of the Nature Publishing Group.)

of protein conformations is the existence of **amino acid networks** that facilitate communication between the different sites and that are linked to the mechanism of catalysis. NMR relaxation dispersion (Section 13.5.2) and isothermal titration calorimetry (Section 15.3.3) studies have shown that the timescale of linked amino acid networks is milliseconds to microseconds and the timescale of binding site change is microseconds to nanoseconds. These values compare with the very fast rate of atomic fluctuations (nanoseconds to picoseconds).

15.3 ANALYTICAL METHODS FOR THE STUDY OF ENZYME REACTIONS

15.3.1 General considerations

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

- to determine the amount (or concentration) of enzyme present in a particular preparation (this is particularly important in diagnostic enzymology, Section 16.3);
- 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};
- to study the effect of pH, temperature, inhibitors, etc. on the enzyme and to make comparative studies of other enzymes that may be involved in a metabolic or signalling pathway of which the enzymes are members. The study of enzyme inhibition is fundamental to the development of new drugs.

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.

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 only over 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 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. 15.11, which represents the effect of enzyme concentration on the progress of the reaction in the presence of a constant initial substrate concentration (Fig. 15.11a). 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. 15.11b. 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.

15.3.2 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

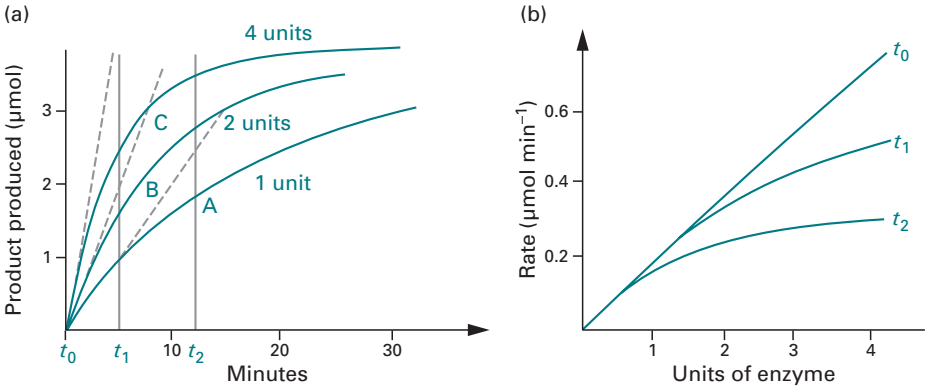
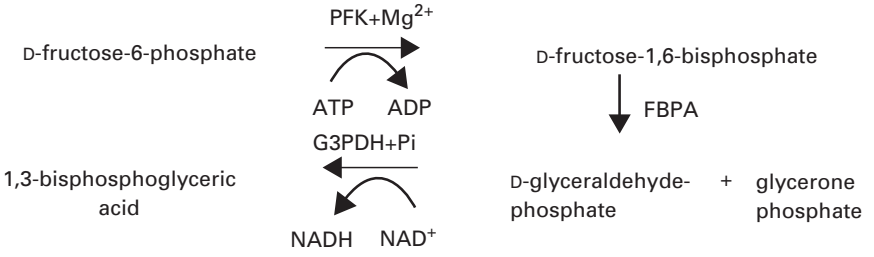


Fig. 15.11 The importance of measuring the initial rate in the assay of an enzyme. (a) Time-dependent variation in the concentration of products in the presence of 1, 2 and 4 units of enzyme; (b) variation of reaction rate with enzyme concentration using true initial rate (v_0) and two fixed time assays (t_1 and t_2).

assay. It is essential that the substrate and product do not absorb at the same wavelength and that the Beer–Lambert law (Section 12.2.2) 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** that 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:

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

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 ($6.3 \times 10^3\ M^{-1}\ cm^{-1}$). By dividing the above equation by the total concentration of protein in the test enzyme preparation, the **specific activity** (katals kg^{-1}) 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 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.

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. 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-methylumbelliferyl-β-D-glucuronide as substrate and assaying 4-methyl-umbelliferone as the fluorescent product. The large commercial interest in the development of inhibitors of kinases, phosphatases and proteases for their therapeutic potential has stimulated the development of assays for these enzymes using fluorogenic substrates to allow kinetic measurements of enzyme activity to be undertaken in vivo using the principle of fluorescence resonance energy transfer (FRET). These substrates contain two fluorochromes situated less than 100 Å apart joined by a 'linker' that is cleaved by the test enzyme and such that the emission wavelength of the donor fluorochrome overlaps with the excitation wavelength of the acceptor fluorochrome allowing the former to transfer energy to the latter. The most commonly used fluors are cyan fluorescent protein, red fluorescent protein and yellow fluorescent protein.

Luminescence methods

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

$$\text{luciferin} + \text{ATP} + \text{O}_2 \overset{\text{luciferase}}{\rightleftharpoons} \text{oxyluciferin} + \text{AMP} + \text{PP}_\text{i} + \text{CO}_2 + \textit{light}$$

The assay can be used to assay ATP and enzymes that utilise ATP by means of coupled reactions. 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 ELISA-based assay for the enzyme. Such assays can distinguish between isoenzyme forms, which make the assay attractive for diagnostic purposes. An important clinical example is creatine kinase. 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.

15.3.3 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. Figure 15.1 shows the progress curves for this pre-steady-state initial stage of an enzymic reaction. The induction time t is related to the rate constants for the formation and dissociation of the 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 preselected 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. 15.12). 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.

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, such as trichloroacetic acid, stops the reaction that is then monitored by an appropriate analytical method for the build-up of intermediates.

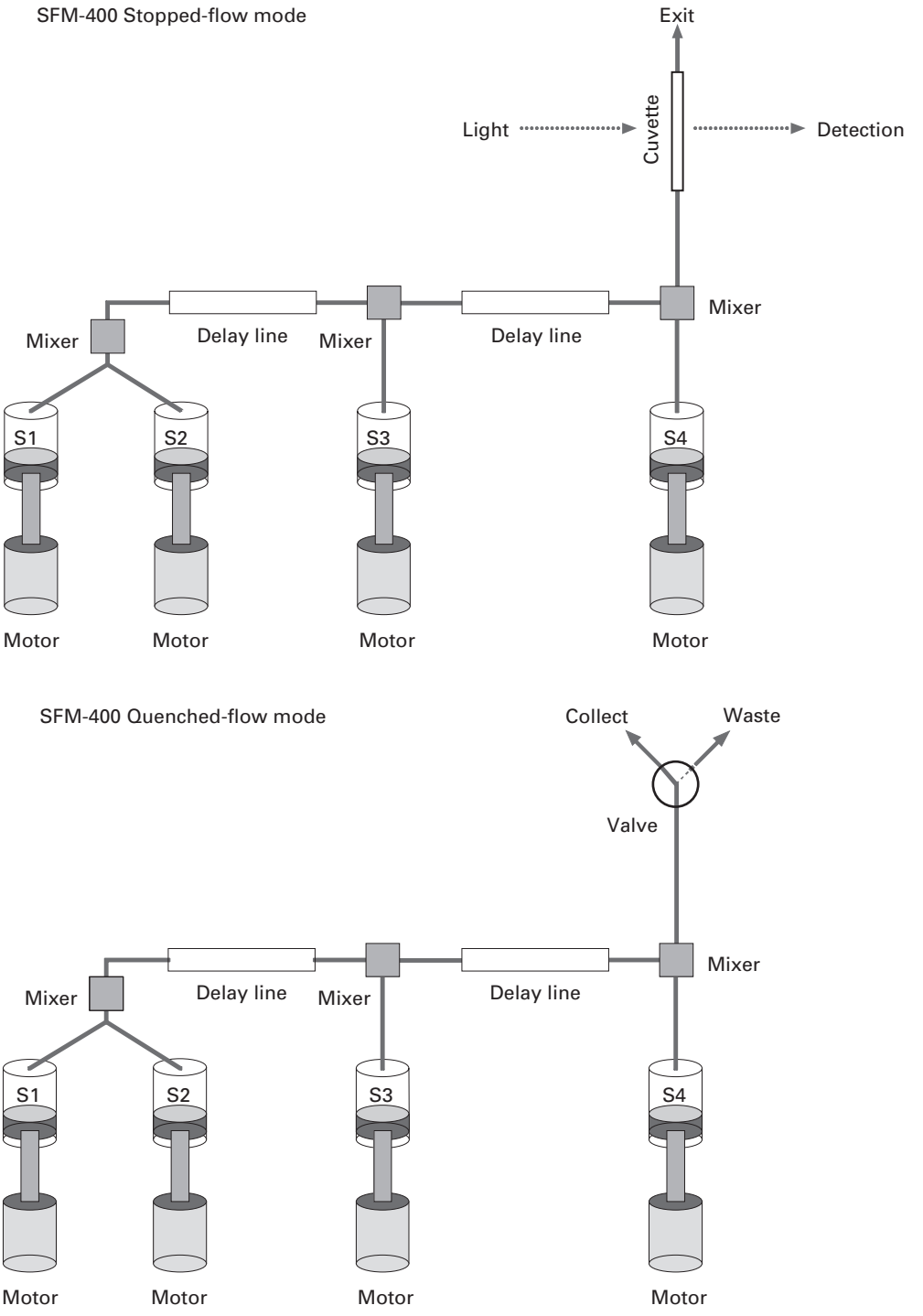


Fig. 15.12 The BioLogic stopped-flow and quenched-flow apparatus. The reactants are placed in separate syringes each driven by a microprocessor-controlled stepping motor capable of delivering 0.01 to 10.00 cm^3 min^{-1} with a minimum injection volume of 10–30 mm^3. The reactants are pre-mixed before they

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 due 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 °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 enable 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 to 10^8 M^{-1} s^{-1} and first-order rate constants for the dissociation of ES in the range 10 to 10^4 s^{-1}. The upper limit of these values is such that for some enzymes virtually every interaction between an enzyme and its substrate leads to the formation of a complex. 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 pathways (Section 17.4.1).

Isothermal titration calorimetry

This is a general method for studying the thermodynamics of any binding (association) process. It detects and quantifies small heat changes associated with the binding and has the advantages of speed, accuracy and not requiring either of the reacting species to be

Caption for fig. 15.12 (*cont.*)
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.)

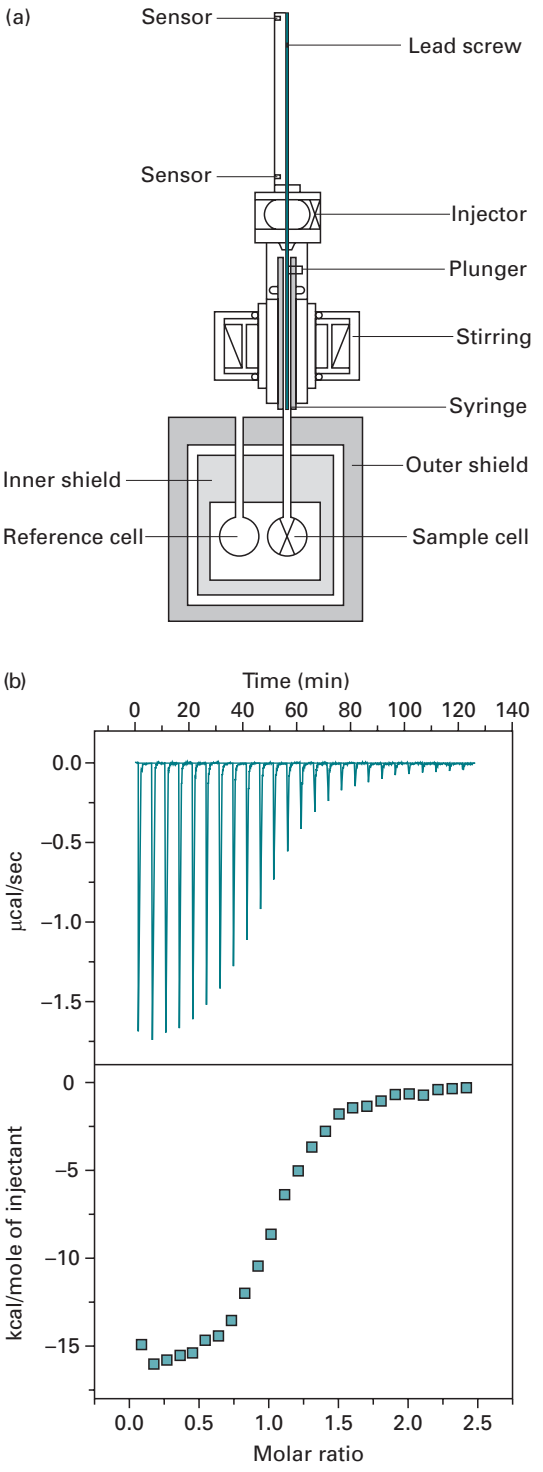


Fig 15.13 Isothermal titration calorimetry. (a) Diagram of MicroCal ITC cell and syringe. A thermoelectric device measures the temperature difference between the two cells and a second device measures the temperature difference between the two cells and the jacket. The plunger is computer-controlled and injects precise volumes

chemically modified or immobilised. The apparatus consists of a pair of matched cells (sample and reference) of approximately 2 cm^3 volume contained in a microcalorimeter (Fig. 15.13a). One of the reactants (say the enzyme preparation) is added to the sample cell and the ligand (substrate, inhibitor or effector) added via a stepper-motor-driven syringe. The mixture is stirred to ensure homogeneity. The reference cell contains an equal volume of reference liquid. A constant power of less than 1 mW is applied to the reference cell. This directs a feedback circuit activating a heater attached to the sample cell. The addition of the ligand solution causes a heat change due to the binding process and the dilution of both the enzyme and ligand preparations. If the reaction is exothermic less energy is required to maintain the cell at constant temperature. If the reaction is endothermic more energy is required. The power required to maintain a constant temperature is recorded as a series of spikes as a function of time (Fig. 15.13b). Each spike is integrated to give μcal s^{-1} and summed to give the total heat exchange per injection. The study is repeated with a series of increasing ligand concentrations and control experiments carried out replacing the ligand with buffer solution to allow the heat exchange (ΔH) associated solely with the addition of ligand to be calculated. A plot is then made of enthalpy change against the molar ratio of the ligand to enzyme. The plot is hyperbolic from which it is possible to calculate enthalpy, free energy, and entropy changes associated with the ligand binding and hence the dissociation constant, K_d, and stoichiometry of binding n. Isothermal titration calorimetry has been successfully used in the study of the thermodynamics of the interconversion of protein conformations and the elucidation of the mechanism of allosterism.

15.3.4 Analytical methods for *in vivo* studies

The increasing importance of genome sequencing studies, particularly in the context of drug development, has stimulated the development of techniques for the study of enzymes in intact cells and whole organisms. *In vitro* methods have the disadvantage that they lead to the disruption of organelles and micro-departments, commonly result in the release of activators or inhibitors and invariably use assay conditions that are not representative of the *in vivo* situation. One of the most successful analytical techniques 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

Caption for fig. 15.13 (*cont.*)
of the ligand whilst the syringe rotates to provide continuous mixing. Heat is added or removed from the sample cell, as appropriate, and the associated power required to maintain constant temperature recorded in units of μcal s^{-1}. (b) Data for the binding of 2′ CMP to RNase. Top panel, energy exchange; bottom panel, the binding isotherm from which the value of n (1), K_d (0.85 μM) and ΔH (16.7 kcal mol^{-1}) can be calculated. (Reproduced by permission of MicroCal Europe, Milton Keynes, UK: website www.microcal.com.)

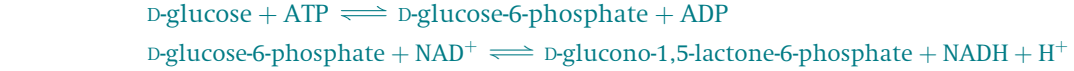
methods is to insert a fluorine atom into the molecule. From an NMR point of view this is an excellent label, since it is a spin-half nuclide that is readily studied by NMR. The chemical shift change of the fluorine nucleus is large, making it very sensitive to its local environment in the protein. Moreover, 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.

15.3.5 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) 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 **enzymic 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:



- *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:

$$\text{D-glucose} + H_2O + O_2 \rightleftharpoons \text{D-gluconic acid} + H_2O_2$$
$$H_2O_2 + \text{dye}_{\text{reduced}} \rightleftharpoons H_2O + \text{dye}_{\text{oxidised}}$$

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. Examples of the commonly used dye are 4-aminophenazine and *o*-dianisidine.

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

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

Another blood substrate commonly assayed by enzyme-based techniques in clinical biochemistry is cholesterol:

- *Cholesterol*: This is used as an indicator of atherosclerosis and susceptibility to coronary heart disease (Section 18.2.2). It uses cholesterol oxidase and peroxidase and measures the absorption in the visible region due to the oxidised dye, for example 4-aminophenazine:

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

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

Biosensors (Sections 1.3.5 and 16.2.2) such as those for glucose and cholesterol are based on the above reactions and afford a simple means for the fast measurement of these substrates.

15.4 ENZYME ACTIVE SITES AND CATALYTIC MECHANISMS

15.4.1 Enzyme active sites

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:

- *X-ray crystallographic studies:* These are capable of giving, either directly or indirectly, decisive information about the mechanism of enzyme action. 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 of effector molecules. The Catalytic Site Atlas (www.ebl.ac.uk/thornton-srv/databases) and Protein Data Bank (www.wwpdp.org) list the active sites and catalytic residues of enzymes whose three-dimensional structure has been determined. 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 (Section 18.2).
- *Irreversible inhibitor and affinity label studies:* Irreversible inhibitors act by forming a covalent bond with the enzyme. 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. 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.
- *Kinetic studies:* This approach is based on the use of a range of substrates and/or competitive inhibitors and the determination of the associated K_m, k_{cat} and K_i values. These allow correlations to be drawn between molecular structure and kinetic constants and hence deductions to be made about the structure of the active site. 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 15.8). 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.

- *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 enables deductions to be made about the mechanism of the reaction. Two examples illustrate the principle. Firstly, alcohol dehydrogenase (AD) that oxidises ethanol to ethanal using NAD^+ : NADH:

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

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 pro*R* 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 only be interpreted 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, the hydrolysis of esters 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 either be retained 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 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.

- *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. 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 to that of the native form.

15.4.2 Catalytic mechanisms

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. The most commonly occurring amino acid residues in enzyme catalytic sites are the imidazole group of histidine, the guanidinium group of arginine, the carboxylate groups of glutamate and aspartate, the amino group of lysine, the hydroxyl groups of serine, threonine and tyrosine and the thiol group of cysteine. Of these nine groups, the imidazole group of histidine appears to be quantitatively the most important. These specific amino acid side chains commonly occur in pairs or triplets within catalytic sites. In the lipases and peptidases the **catalytic triad** serine, aspartate and histidine occurs very commonly. In chymotrypsin for example, the three amino acids are located in positions 195, 102 and 95 respectively and are brought into juxtaposition by the three-dimensional folding of the protein chain. Their roles include:

- to activate the substrate by forming a hydrogen bond thereby lowering the reaction activation energy barrier. Such a hydrogen bond has been referred to as a **low-barrier hydrogen bond**;
- to provide a nucleophile to attack the substrate;
- to provide the components for the acid–base catalysis of the substrate of the type well known in conventional organic chemistry;
- to stabilise the transition state of the reaction.

The quantitative importance of histidine can be explained by the facts that it is the only residue that has a pK_a near neutral so that it can easily function as an acid–base catalyst, and that it can also easily function as a nucleophile and use its charged form to stabilise transition states. Whilst these amino acid side chains provide the components for catalysis, the specificity of the reaction is determined by the three-dimensional structure of the enzyme and the microenvironment it creates within the active site.

The experimental techniques discussed above have successfully identified key amino acids involved in the catalytic process. However, the question remains as to the nature of the factors limiting the rate of catalysis. Recent studies, particularly

those based on NMR relaxation dispersion experiments, have provided clear evidence that a key to the rate of catalysis is the interconversion of a population of protein conformations (Section 15.2.4). For example, studies with the enzyme dihydrofolate reductase have identified five conformations involved in the catalytic process that interconvert at rates that are identical to those previously determined by conventional kinetic experiments. Similarly, studies with the enzyme triosephosphate isomerase have shown that the opening and closing of a loop in the catalytic site has a vital role in the catalytic cycle. The loop has been shown to open and close at a rate of $10\,000\,s^{-1}$, a value that is the same as the catalytic rate constant. This correlation between rate-limiting steps in catalysis and rates of conformational change suggests that as the substrates bind and are converted to products, the population of conformations adjust and act to drive catalysis along the product pathway.

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 a $(\alpha\beta)_2$ 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. The tunnels protect reactive intermediates from coming in 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.

Closely related to multienzyme complexes are the megasynthases responsible for the synthesis of antibiotics such as penicillin and vancomycin. They are large multifunctional enzymes consisting of clusters of active sites known as **modules**. They promote a series of reactions starting with simple organic intermediates that are progressively converted to the antibiotic. During the synthetic process, the intermediates are tethered to carrier proteins that shuttle them in sequence to the designated active site (referred to as **client enzymes**) in the module. NMR evidence indicates that the enzymes and the carrier proteins exist in an ensemble of dynamic conformations and that successful docking between the two to promote the next synthetic stage relies on the selection of the correct conformation in which the enzyme's carrier protein binding site is exposed.

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 basic ways:

- by alteration of the kinetic conditions under which the enzyme is operating;
- by alteration of the amount of the active form of the enzyme present by promoting enzyme synthesis, enzyme degradation or the chemical modification of the enzyme.

The latter option is inherently a long-term one 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 product produced by the enzyme 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. 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. A good example is the regulation of 6-phosphofructokinase discussed earlier.
- *Reversible covalent modification:* This may involve adenylation of a Tyr 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 Tyr, Ser or Thr 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 characteristic feature of many of these kinases is that they are involved in a cascade of enzyme reactions such as glycogenolysis and glycogenesis that 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 electron transport.

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 conditions as near as possible to those found *in vivo* and such that the enzyme is saturated with substrate (i.e. such that $[S] > 10\,K_m$);
- studies to determine whether or not each individual enzyme stage operates at or near equilibrium *in vivo*;
- studies to determine the **flux control coefficient, *C*,** for each enzyme. This is a property of the enzyme that expresses how the flux of reactants through a pathway is influenced by a change in the activity (note: *not* concentration) of the enzyme under the prevailing physiological conditions. Such a change may be induced by allosteric activators or inhibitors or by feedback inhibition. 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 0 means that the flux is not influenced by changes in the activity of that enzyme. The sum of the *C* values for all the enzymes in a given pathway is 1 so that the higher a given *C* value the greater the impact of that enzyme on the flux through the pathway. These *C* values are therefore highest for the rate-determining enzymes.

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.

The application of these three tests to the enzymes in the glycolytic pathway shows that three of the ten enzymes, hexokinase, 6-phosphofructokinase and pyruvate kinase, have a potential rate-limiting role 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 of their activity and all contribute to the control of flux through the glycolytic pathway. 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.

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.

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 disturbing the equilibrium away from the 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 adenylate kinase which catalyses the reaction:

$$2ADP \rightleftharpoons ATP + AMP$$

ATP is normally present in a cell at much higher concentrations that 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 that 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) which is part of the gluconeogenesis pathway from pyruvate to glucose (Fig. 15.14). Both reactions are strongly exergonic and essentially irreversible. Whereas 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 two opposing pathways. PFK converts D-fructose-6-phosphate to D-fructose-1,6-bisphosphate and simultaneously converts ATP to ADP, while 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 adenylate 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.

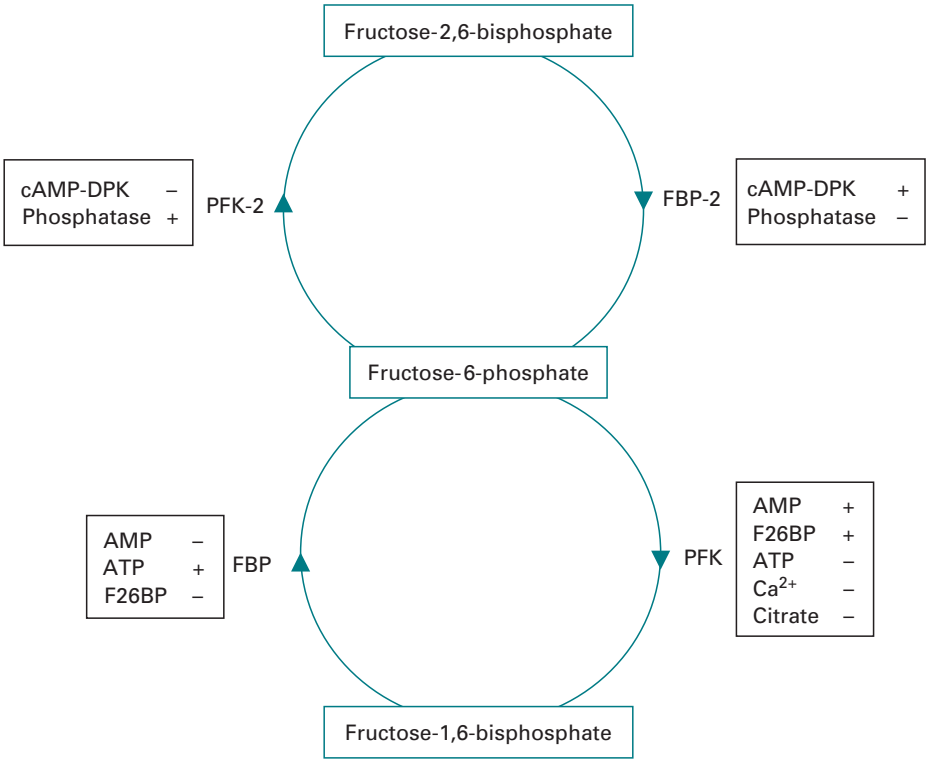


Fig. 15.14 Regulation of phosphofructokinase (PFK). Two substrate cycles each centred on fructose-6-phosphate are involved. Different enzymes promote the forward and reverse reactions of each cycle so that all reactions are exergonic (negative free energy changes). Each enzyme is subject to activation (+) or inhibition (–) either by allosteric effectors or by phosphorylation/dephosphorylation (cAMP-DPK, cyclic AMP-dependent protein kinase). The importance of each regulatory mechanism varies between organisms and between different tissues in a given organism.

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 enabled opposing pathways to be controlled and small changes in the concentration of ATP to be amplified in terms of concomitant changes in AMP that 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, cAMP-dependent protein kinase, phosphorylase kinase, phosphorylase and glycogen. cAMP released from adenylate cyclase as a result of its activation by a G_s protein (see Section 17.4.3) activates cAMP-dependent protein kinase, which in its inactive form is a tetramer consisting of two regulatory (R) and two catalytic (C) subunits (R_2C_2). 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 and it is this that acts as a kinase that in the presence of ATP phosphorylates and thereby activates phosphorylase kinase:

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

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 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+} ions to the δ subunit. The binding of Ca^{2+} ions to the δ subunit promotes the autophosphorylation of the enzyme at a different site to 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 a different induction mechanism to 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. 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 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. As is discussed in more detail in Section 17.4.4, receptor-linked cascades seldom operate in isolation but rather form intricate networks that better allow the fine control necessary for the maintenance of homeostasis.

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 timescale. 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 $K_i = 10^{-13}$ M and a binding rate constant of 10^7 M^{-1} 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 acids), 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 monoubiquitinate or polyubiquitinate a protein and the functional consequences vary. Monoubiquitination leads to the 'trafficking' of the protein, a process that is fundamental to the cycling of receptors (Section 17.5.2), whereas polyubiquitination leads to degradation. More than 12 ubiquitin-binding domains have been identified on proteins but they all bind to the same hydrophobic patch of ubiquitin which contains Ile44 as a central residue. There is increasing evidence that proteins to be degraded contain specific degradation signals, referred to as **degrons**, and that in some cases these signals are controlled by the protein folding or assembly so that biosynthetic errors and misfolding can be recognised and the protein removed by degradation. Misfolded proteins are ubiquitinated and directed to a juxtanuclear intracellular compartment where proteasomes (see below) are also concentrated.

The interaction between ubiquitin and a protein involves a series of enzymes and stages (Fig 15.15):

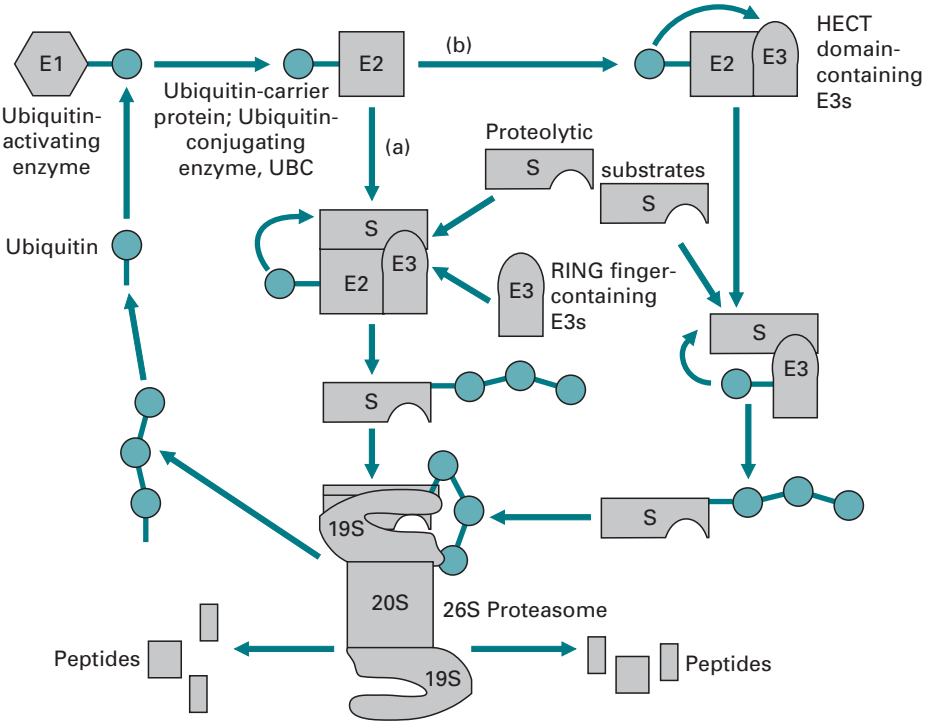


Fig.15.15 The ubiquitin–proteasome system (UPS). Ubiquitin is first activated to a high-energy intermediate by E1. It is then transferred to a member of the E2 family of enzymes. From E2 it can be transferred directly to the substrate (S) that is bound specifically to a member of the ubiquitin ligase family of proteins, E3 (a). This occurs when the E3 belongs to the RING finger family of ligases. In the case of a HECT-domain-containing ligase (b), the activated ubiquitin is transferred first to the E3 before it is conjugated to the E3-bound substrate. Additional ubiquitin moieties are added successively to the previously conjugated moiety to generate a polyubiquitin chain. The polyubiquitinated substrate binds to the 26S proteasome complex, the substrate is degraded to short peptides, and free and reusable ubiquitin is released through the activity of deubiquitinating enzymes (DUBs). (Reproduced from A. Ciechanover and R. Ben-Saadon (2004). N-terminal ubiquitination: more protein substrates join in. *Trends in Cell Biology*, **14**, 103–106, by permission of Elsevier Science.)

- *Ubiquitin-activating enzyme* (E1s): This requires ATP and involves the initial formation of a ubiquitin–adenylate intermediate. Ubiquitin is then transferred to a cysteine residue in the active site of the E1 with the concomitant release of AMP.
- *Ubiquitin-conjugating enzymes* (E2s): These receive the ubiquitin from the E1 by a transthioesterification reaction. 20–30 different E2s are known.
- *Ubiquitin protein ligases* (E3s): These interacts with both the E2 and the substrate by means of either a HECT or RING binding domain on the E3 resulting in the transfer of the ubiquitin to a lysine residue in the substrate. Results from the Human Genome Project provide evidence for the existence of several hundred of these E3 enzymes.
- The process either stops after monoubiquitination or is repeated resulting in the attachment of four or more ubiquitin residues to the substrate, a process called **polyubiquitination**.

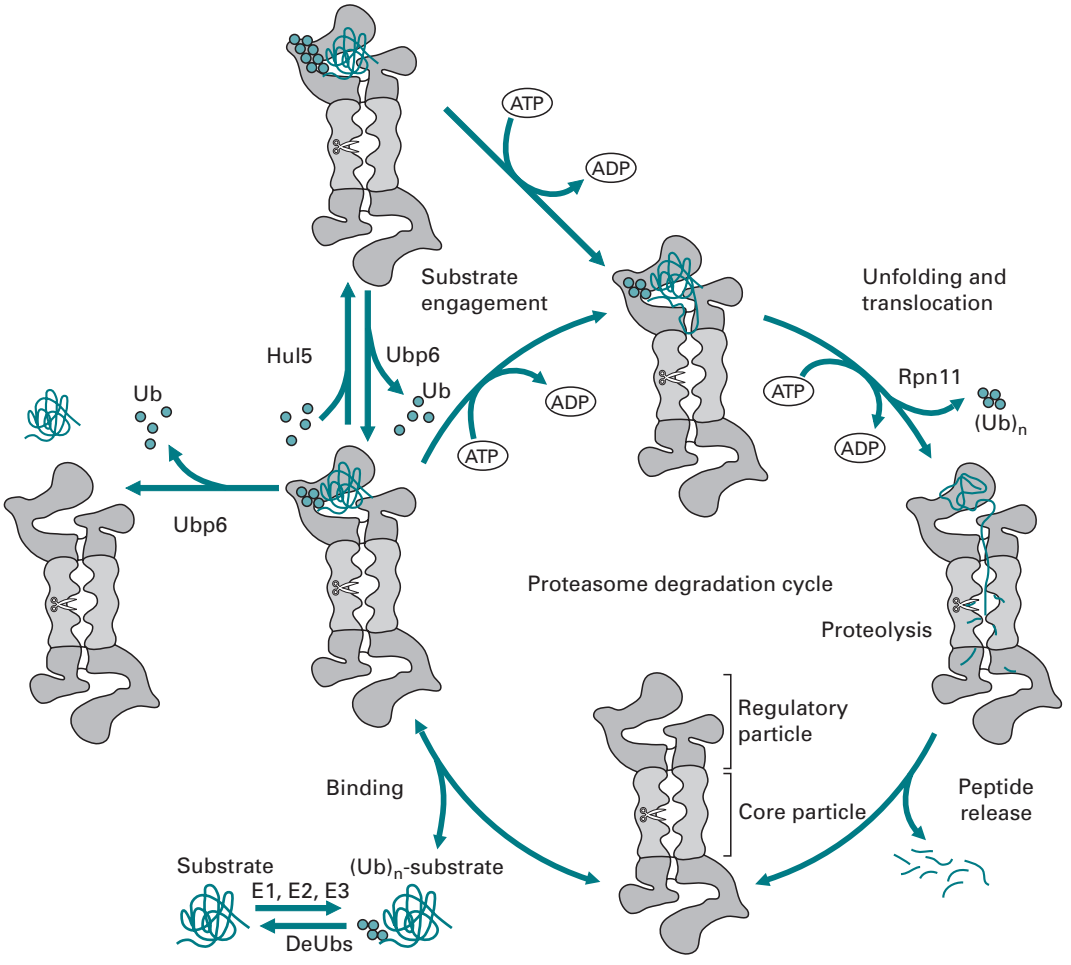


Fig. 15.16 Proteasome-degrading cycle, incorporating Hul5 and Ubp6 activities. Substrates are polyubiquitinated through the action of E1, E2 and E3 enzymes. Following binding to the proteasome, substrates are either engaged, unfolded and degraded in an ATP-dependent manner, with concomitant removal of the polyubiquitin chain by Rpn11, or are released from the proteasome. Release can be accelerated through the deubiquitinating action of Ubp6, which decreases substrate affinity. Further elongation of the ubiquitin chain by Hul5 increases affinity of the substrate for the proteasome, decreasing the release rate and thereby increasing degradation. The balance of Ubp6 and Hul5 activities can therefore affect the partitioning between binding and release by modifying the polyubiquitin chain length and thereby affecting the release rate. (Reproduced from D. A. Kraut, S. Prakash and A. Matouschek (2007). To degrade or release: ubiquitin-chain remodelling. *Trends in Cell Biology*, **17**, 419–421, by permission of Elsevier Science.)

The polyubiquitinated substrate is degraded by a multicatalytic complex based on a 20S 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 20S 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

may either 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 19S regulatory complex unit that consists of approximately 20 subunits, six of which have ATPase sites. These ATPases have similar structures but distinct functions that include the capture of the protein to be degraded, unfolding its structure and injection into the proteasome. This 19S complex unit combines with both ends of the 20S proteasome cylinder to form a 26S proteasome that promotes the cleavage of the peptide bonds with the concomitant hydrolysis of ATP. The recruitment and degradation of a protein relies on the presence of two subunits, Rpn1 and Rpn2, in the 19S unit. Two other proteins, Hul5 and Ubp6, when bound to the proteasome also regulate the degradation process (Fig. 15.16).

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 and cell cycle control. Dysfunction of the ubiquitin–proteasome pathway has been implicated in a number of disease states. For example, there is evidence that the accumulation of abnormal or damaged proteins due to impairment of the pathway contributes to a number of neurodegenerative diseases including Alzheimer's. In contrast, deliberately blocking the pathway in cancer cells could lead to a disruption of protein regulation that in turn could cause the apoptosis of the malignant cells. Accordingly, proteasome inhibitors have been developed for evaluation as anti-tumour agents against selected cancers. Bortezomib is one such inhibitor that targets the 26S proteasome, and in combination with other chemotherapeutic agents was shown to have therapeutic potential.

15.6 SUGGESTIONS FOR FURTHER READING

General texts

Frey, P. and Hegeman, A. (2007). *Enzymatic Reaction Mechanisms*. Oxford: Oxford University Press. (Discusses over 100 case studies of enzyme mechanisms.)

Review articles

Barglow, K. T. and Cravatt, B. F. (2007). Activity-based protein profiling for the functional annotation of enzymes. *Nature Methods*, **4**, 822–827.

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Furnham, N., Garavelli, J. S., Apweiler, R. and Thornton, J. M. (2009). Missing in action: enzyme functional annotations in biological databases. *Nature Chemical Biology*, **5**, 521–525.

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Zalatan, J. G. and Herschlag, D. (2009). The far reaches of enzymology. *Nature Chemical Biology*, **5**, 516–520.