CHAPTER 10

Enzyme Kinetics

One of the most fascinating areas of study in chemical kinetics is enzyme catalysis. The phenomenon of enzyme catalysis usually results in a very large increase in reaction rate (on the order of 10^6 to 10^{18}) and high specificity. By specificity, we mean that an enzyme molecule is capable of selectively catalyzing certain reactants, called *substrates*, while discriminating against other molecules.

This chapter presents the basic mathematical treatment of enzyme kinetics and discusses the topics of enzyme inhibition, allosterism, and the effect of pH on enzyme kinetics.

10.1 General Principles of Catalysis

A *catalyst* is a substance that increases the rate of a reaction without itself being consumed by the process. A reaction in which a catalyst is involved is called a *catalyzed reaction*, and the process is called *catalysis*. In studying catalysis, keep in mind the following characteristics:

1. A catalyst lowers the Gibbs energy of activation by providing a different mechanism for the reaction (Figure 10.1). This mechanism enhances the rate and it applies to *both* the forward and the reverse directions of the reaction.

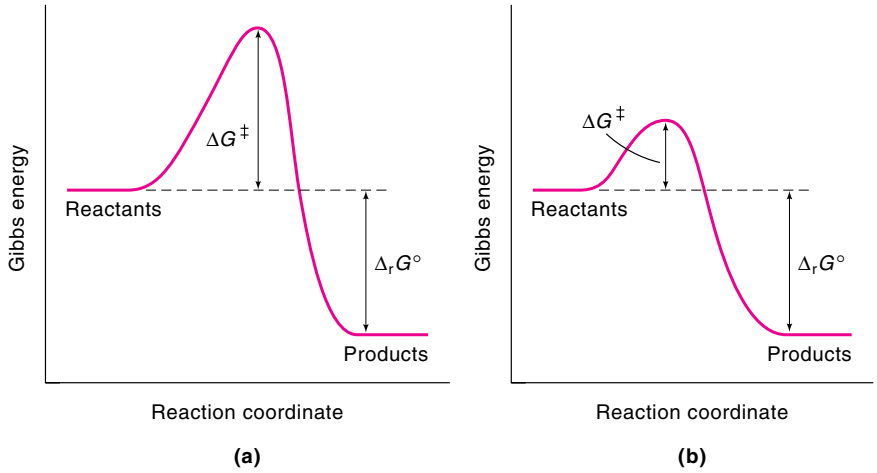


Figure 10.1
Gibbs energy change for (a) an uncatalyzed reaction and (b) a catalyzed reaction. A catalyzed reaction must involve the formation of at least one intermediate (between the reactant and the catalyst). The $\Delta_r G°$ is the same in both cases.

2. A catalyst forms an intermediate with the reactant(s) in the initial step of the mechanism and is released in the product-forming step. The catalyst does not appear in the overall reaction.
3. Regardless of the mechanism and the energetics of a reaction, a catalyst cannot affect the enthalpies or Gibbs energies of the reactants and products. Thus, catalysts increase the rate of approach to equilibrium, but cannot alter the thermodynamic equilibrium constant.

Humans have used catalysts for thousands of years in food preparation and wine making. Industrially, hundreds of billions of dollars worth of chemicals are produced annually with the aid of catalysts. There are three types of catalysis: heterogeneous, homogeneous, and enzymatic. In a heterogeneously catalyzed reaction, the reactants and the catalyst are in different phases (usually gas/solid or liquid/solid). Well-known examples are the Haber synthesis of ammonia and the Ostwald manufacture of nitric acid. The bromination of acetone, catalyzed by acids,

$$CH_3COCH_3 + Br_2 \xrightarrow{H^+} CH_2BrCOCH_3 + HBr$$

is an example of homogeneous catalysis because the reactants and the catalyst (H^+) are all present in the aqueous medium. Enzyme catalysis is also mostly homogeneous in nature. However, because it is of biological origin and is the most complex of the three types of catalysis, enzyme catalysis is treated as a separate category. Whether or not their mechanisms are well understood, enzymes have been used widely in food and beverage production, as well as in the manufacture of drugs and other chemicals.

Enzyme Catalysis

Since 1926, when the American biochemist James Sumner (1887–1955) crystallized urease (an enzyme that catalyzes the cleavage of urea to ammonia and carbon dioxide), it has come to be known that most enzymes are proteins.* An enzyme usually contains one or more *active sites*, where reactions with substrates take place. An active site may comprise only a few amino acid residues; the rest of the protein is required for maintaining the three-dimensional integrity of the network. The specificity of enzymes for substrates varies from molecule to molecule. Many enzymes exhibit stereochemical specificity in that they catalyze the reactions of one conformation but not the other (Figure 10.2). For example, proteolytic enzymes catalyze only

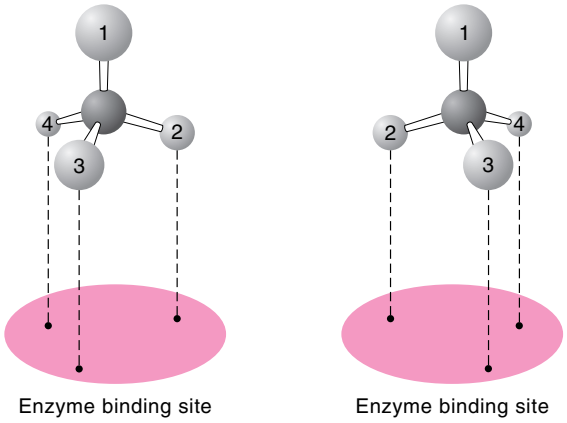


Figure 10.2
Diagram showing how two enantiomers bind differently to an enzyme. Because the geometry of an enzyme's active site is normally fixed (that is, it can have only one of the above two arrangements), a reaction occurs for only one of the two enantiomers. Specificity requires a minimum of three contact points between the substrate and the enzyme.

* In the early 1980s, chemists discovered that certain RNA molecules, called ribozymes, also possess catalytic properties.

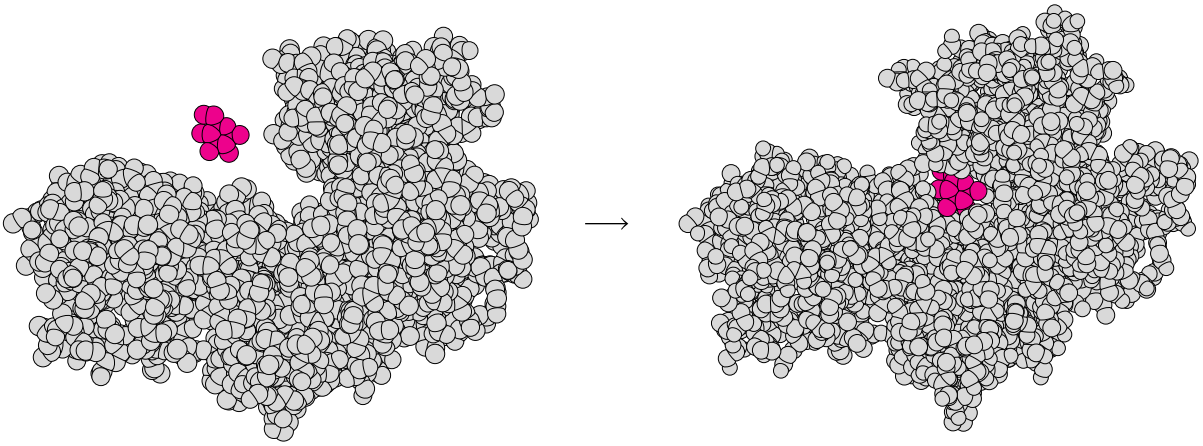


Figure 10.3
The conformational change that occurs when glucose binds to hexokinase, which is an enzyme in the metabolic pathway. [From W. S. Bennet and T. A. Steitz, *J. Mol. Biol.* **140**, 211 (1980).]

the hydrolysis of peptides made up of L-amino acids. Some enzymes are catalytically inactive in the absence of certain metal ions.

In the 1890s the German chemist Emil Fischer (1852–1919) proposed a lock-and-key theory of enzyme specificity. According to Fischer, the active site can be assumed to have a rigid structure, similar to a lock. A substrate molecule then has a complementary structure and functions as a key. Although appealing in some respects, this theory has been modified to take into account the flexibility of proteins in solution. We now know that the binding of the substrate to the enzyme results in a distortion of the substrate into the conformation of the transition state. At the same time, the enzyme itself also undergoes a change in conformation to fit the substrate (Figure 10.3). The flexibility of the protein also explains the phenomenon of cooperativity. *Cooperativity* means the binding of a substrate to an enzyme with multiple binding sites can alter the substrate's affinity for enzyme binding at its other sites.

Enzymes, like other catalysts, increase the rate of a reaction. An understanding of the efficiency of enzymes can be gained by examining Equation 9.41:

$$k = \frac{k_B T}{h} e^{-\Delta G^{\circ\ddagger}/RT} (M^{1-m})$$

$$= \frac{k_B T}{h} e^{\Delta S^{\circ\ddagger}/R} e^{-\Delta H^{\circ\ddagger}/RT} (M^{1-m})$$

There are two contributions to the rate constant: $\Delta H^{\circ\ddagger}$ and $\Delta S^{\circ\ddagger}$. The enthalpy of activation is approximately equal to the energy of activation (E_a) in the Arrhenius equation (see Equation 9.28). Certainly a reduction in E_a by the action of a catalyst would enhance the rate constant. In fact, this is usually the explanation of how a catalyst works, but it is not always true for enzyme catalysis. Entropy of activation, $\Delta S^{\circ\ddagger}$, may also be an important factor in determining the efficiency of enzyme catalysis.

Consider the bimolecular reaction

$$A + B \rightarrow AB^{\ddagger} \rightarrow \text{product}$$

where A and B are both nonlinear molecules. Before the formation of the activated complex, each A or B molecule has three translational, three rotational, and three vibrational degrees of freedom. These motions all contribute to the entropy of the molecule. At 25°C, the greatest contribution comes from translational motion (about 120 J K^{-1} mol^{-1}), followed by rotational motion (about 80 J K^{-1} mol^{-1}). Vibrational motion makes the smallest contribution (about 15 J K^{-1} mol^{-1}). The translational and rotational entropies of the activated complex are only slightly larger than those of an individual A or B molecule (these entropies increase slowly with size); therefore, there is a net loss in entropy of about 200 J K^{-1} mol^{-1} when the activated complex is formed. This loss in entropy is compensated for to a small extent by new modes of internal rotation and vibration in the activated complex. For unimolecular reactions, such as the *cis–trans* isomerization of an alkene, however, there is very little entropy change because the activated complex is formed from a single molecular species. A theoretical comparison of a unimolecular reaction with a bimolecular one shows a difference of as much as 3×10^{10} in the $e^{\Delta S^{\circ\ddagger}/R}$ term, favoring the unimolecular reaction.

Consider a simple enzyme-catalyzed reaction in which one substrate (S) is transformed into one product (P). The reaction proceeds as follows:

$$E + S \rightleftharpoons ES \rightleftharpoons ES^{\ddagger} \rightleftharpoons EP \rightleftharpoons E + P$$

In this scheme, the enzyme and the substrate must first encounter each other in solution to form the enzyme–substrate intermediate, ES. This is a reversible reaction but when [S] is high, the formation of ES is favored. When the substrate is bound, forces within the active site can align the substrate and enzyme reactive groups into proper orientation, leading to the activated complex. The reaction takes place in the single entity enzyme–substrate intermediate to form the enzyme–substrate activated complex ($ES^{\ddagger}$), as in a unimolecular reaction, so the loss in entropy will be much less. In other words, the loss of the translational and rotational entropies occurred during the formation of ES, and not during the ES → $ES^{\ddagger}$ step. (This loss of entropy is largely compensated for by the substrate binding energy.) Once formed, $ES^{\ddagger}$ proceeds energetically downhill to the enzyme–product intermediate and finally to the product with the regeneration of the enzyme. Figure 10.4 summarizes the steps on a diagram of Gibbs energy versus reaction coordinate.

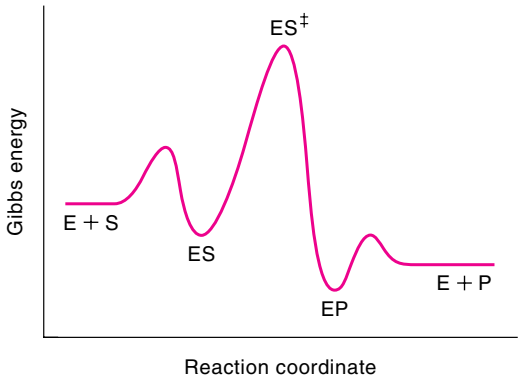


Figure 10.4
Plot of Gibbs energy versus reaction coordinate for an enzyme-catalyzed reaction.

10.2 The Equations of Enzyme Kinetics

In enzyme kinetics, it is customary to measure the *initial rate* (v_0) of a reaction to minimize reversible reactions and the inhibition of enzymes by products. Further-

more, the initial rate corresponds to a known fixed substrate concentration. As time proceeds, the substrate concentration will drop.

Figure 10.5 shows the variation of the initial rate (v_0) of an enzyme-catalyzed reaction with substrate concentration [S], where the subscript zero denotes the initial value. The rate increases rapidly and linearly with [S] at low substrate concentrations, but it gradually levels off toward a limiting value at high concentrations of the substrate. In this region, all the enzyme molecules are bound to the substrate molecules, and the rate becomes zero order in substrate concentration. Mathematical analysis shows that the relationship between v_0 and [S] can be represented by an equation of a rectangular hyperbola:

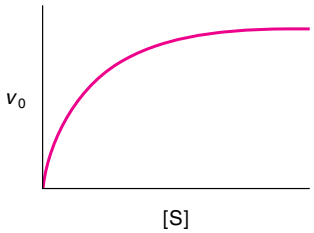


Figure 10.5
Plot of the initial rate of an enzyme-catalyzed reaction versus substrate concentration.

$$v_0 = \frac{a[\mathrm{S}]}{b + [\mathrm{S}]} \tag{10.1}$$

where a and b are constants. Our next step is to develop the necessary equations to account for the experimental data.

Michaelis–Menten Kinetics

In 1913, the German biochemist Leonor Michaelis (1875–1949) and the Canadian biochemist Maud L. Menten (1879–1960), building on the work of the French chemist Victor Henri (1872–1940), proposed a mechanism to explain the dependence of the initial rate of enzyme-catalyzed reactions on concentration. They considered the following scheme, in which ES is the enzyme–substrate complex:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{P} + \mathrm{E}$$

The initial rate of product formation, v_0, is given by

$$v_0 = \left(\frac{d[\mathrm{P}]}{dt}\right)_0 = k_2[\mathrm{ES}] \tag{10.2}$$

To derive an expression for the rate in terms of the more easily measurable substrate concentration, Michaelis and Menten assumed that $k_{-1} \gg k_2$ so that the first step (formation of ES) can be treated as a rapid equilibrium process. The dissociation constant, K_S, is given by

This step corresponds to the pre-equilibrium case discussed on p. 331.

$$K_\mathrm{S} = \frac{k_{-1}}{k_1} = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]}$$

The total concentration of the enzyme at a time shortly after the start of the reaction is

$$[\mathrm{E}]_0 = [\mathrm{E}] + [\mathrm{ES}]$$

so that

$$K_\mathrm{S} = \frac{([\mathrm{E}]_0 - [\mathrm{ES}])[\mathrm{S}]}{[\mathrm{ES}]} \tag{10.3}$$

Solving for [ES], we obtain

$$[\mathrm{ES}] = \frac{[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S} + [\mathrm{S}]} \tag{10.4}$$

Substituting Equation 10.4 into Equation 10.2 yields

$$v_0 = \left(\frac{d[\mathrm{P}]}{dt}\right)_0 = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S} + [\mathrm{S}]} \tag{10.5}$$

Thus, the rate is always proportional to the total concentration of the enzyme.

Equation 10.5 has the same form as Equation 10.1, where $a = k_2[\mathrm{E}]_0$ and $b = K_\mathrm{S}$. At low substrate concentrations $[\mathrm{S}] \ll K_\mathrm{S}$, so Equation 10.5 becomes $v_0 = (k_2/K_\mathrm{S})[\mathrm{E}]_0[\mathrm{S}]$; that is, it is a second-order reaction (first order in $[\mathrm{E}]_0$ and first order in [S]). This rate law corresponds to the initial linear portion of the plot in Figure 10.5. At high substrate concentrations, $[\mathrm{S}] \gg K_\mathrm{S}$, so Equation 10.5 can be written

$$v_0 = \left(\frac{d[\mathrm{P}]}{dt}\right)_0 = k_2[\mathrm{E}]_0$$

Under these conditions, all the enzyme molecules are in the enzyme–substrate complex form; that is, the reacting system is saturated with S. Consequently, the initial rate is zero order in [S]. This rate law corresponds to the horizontal portion of the plot. The curved portion in Figure 10.5 represents the transition from low to high substrate concentrations.

When all the enzyme molecules are complexed with the substrate as ES, the measured initial rate must be at its maximum value (V_max), so that

$$V_\mathrm{max} = k_2[\mathrm{E}]_0 \tag{10.6}$$

where V_max is called the *maximum rate*. Now consider what happens when $[\mathrm{S}] = K_\mathrm{S}$. From Equation 10.5 we find that this condition gives $v_0 = V_\mathrm{max}/2$, so K_S equals the concentration of S when the initial rate is half its maximum value.

Steady-State Kinetics

The British biologists George Briggs (1893–1978) and John Haldane (1892–1964) showed in 1925 that it is unnecessary to assume that enzyme and substrate are in thermodynamic equilibrium with the enzyme–substrate complex to derive Equation 10.5. They postulated that soon after enzyme and substrate are mixed, the concentration of the enzyme–substrate complex will reach a constant value so that we can apply the steady-state approximation as follows (Figure 10.6):*

$$\begin{aligned}\frac{d[\mathrm{ES}]}{dt} = 0 &= k_1[\mathrm{E}][\mathrm{S}] - k_{-1}[\mathrm{ES}] - k_2[\mathrm{ES}] \\ &= k_1([\mathrm{E}]_0 - [\mathrm{ES}])[\mathrm{S}] - (k_{-1} + k_2)[\mathrm{ES}]\end{aligned}$$

Solving for [ES], we get

$$[\mathrm{ES}] = \frac{k_1[\mathrm{E}]_0[\mathrm{S}]}{k_1[\mathrm{S}] + k_{-1} + k_2} \tag{10.7}$$

* Chemists are also interested in *pre-steady-state kinetics*—that is, the period before steady state is reached. Pre-steady-state kinetics is more difficult to study but provides useful information regarding the mechanism of enzyme catalysis. But steady-state kinetics is more important for the understanding of metabolism, because it measures the rates of enzyme-catalyzed reactions in the steady-state conditions that exist in the cell.

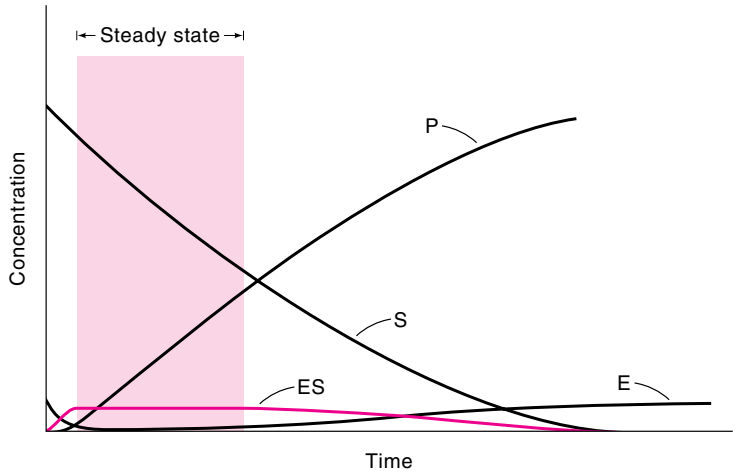


Figure 10.6
Plot of the concentrations of the various species in an enzyme-catalyzed reaction $E + S \rightleftharpoons ES \rightarrow P + E$ versus time. We assume that the initial substrate concentration is much larger than the enzyme concentration and that the rate constants k_1, k_{-1}, and k_2 (see text) are of comparable magnitudes.

Substituting Equation 10.7 into 10.2 gives

$$v_0 = \left(\frac{d[P]}{dt}\right)_0 = k_2[ES] = \frac{k_1 k_2 [E]_0 [S]}{k_1[S] + k_{-1} + k_2}$$
$$= \frac{k_2[E]_0[S]}{[(k_{-1} + k_2)/k_1] + [S]}$$
$$= \frac{k_2[E]_0[S]}{K_M + [S]} \qquad (10.8)$$

where K_M, the *Michaelis constant*, is defined as

$$K_M = \frac{k_{-1} + k_2}{k_1} \qquad (10.9)$$

Comparing Equation 10.8 with Equation 10.5, we see that they have a similar dependence on substrate concentration; however, $K_M \neq K_S$ in general unless $k_{-1} \gg k_2$.

The Briggs–Haldane treatment defines the maximum rate exactly as Equation 10.6 does. Because $[E]_0 = V_{max}/k_2$, Equation 10.8 can also be written as

$$v_0 = \frac{V_{max}[S]}{K_M + [S]} \qquad (10.10)$$

Equation 10.10 is a fundamental equation of enzyme kinetics, and we shall frequently refer to it. When the initial rate is equal to half the maximum rate, Equation 10.10 becomes

$$\frac{V_{max}}{2} = \frac{V_{max}[S]}{K_M + [S]}$$

or

$$K_M = [S]$$

Note that the larger the K_M (the weaker the binding), the larger the [S] needed to reach the half maximum rate.

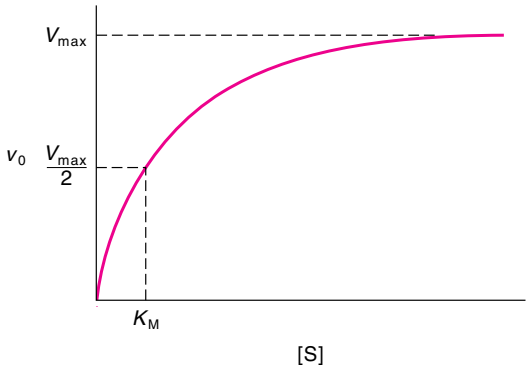


Figure 10.7
Graphical determination of V_{max} and K_M.

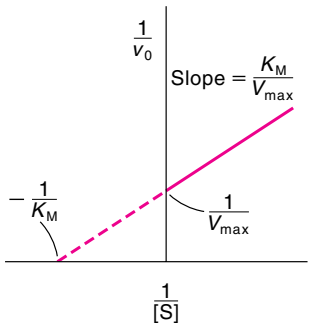


Figure 10.8
Lineweaver–Burk plot for an enzyme-catalyzed reaction obeying Michaelis–Menten kinetics.

Thus, both V_{max} and K_M can be determined, at least in principle, from a plot such as the one in Figure 10.7. In practice, however, we find that the plot of v_0 versus [S] is not very useful in determining the value of V_{max} because locating the asymptotic value V_{max} at very high substrate concentrations is often difficult. A more satisfactory approach, suggested by the American chemists H. Lineweaver (1907–) and Dean Burk (1904–1988), is to employ the double-reciprocal plot of $1/v_0$ versus $1/[S]$. From Equation 10.10, we write

$$\frac{1}{v_0} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \tag{10.11}$$

As Figure 10.8 shows, both K_M and V_{max} can be obtained from the slope and intercepts of the straight line.

Although useful and widely employed in enzyme kinetic studies, the Lineweaver–Burk plot has the disadvantage of compressing the data points at high substrate concentrations into a small region and emphasizing the points at lower substrate concentrations, which are often the least accurate. Of the several other ways of plotting the kinetic data, we shall mention the Eadie–Hofstee plot. Multiplying both sides of Equation 10.11 by $v_0 V_{max}$, we obtain

$$V_{max} = v_0 + \frac{v_0 K_M}{[S]}$$

Rearrangement gives

$$v_0 = V_{max} - \frac{v_0 K_M}{[S]} \tag{10.12}$$

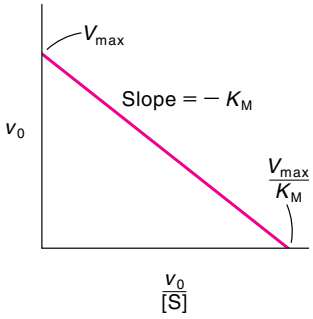


Figure 10.9
Eadie–Hofstee plot for the reaction graphed in Figure 10.7.

This equation shows that a plot of v_0 versus $v_0/[S]$, the so-called Eadie–Hofstee plot, gives a straight line with slope equal to $-K_M$ and intercepts V_{max} on the v_0 axis and V_{max}/K_M on the $v_0/[S]$ axis (Figure 10.9).

The Significance of K_M and V_{max}

The Michaelis constant, K_M, varies considerably from one enzyme to another, and also with different substrates for the same enzyme. By definition, it is equal to the substrate concentration at half the maximum rate. Put another way, K_M represents the substrate concentration at which half the enzyme active sites are filled by substrate molecules. The value of K_M is sometimes equated with the dissociation con-

stant of the enzyme–substrate complex, ES (the larger the K_M, the weaker the binding). As can be seen from Equation 10.9, however, this is true only when $k_2 \ll k_{-1}$ so that $K_M = k_{-1}/k_1$. In general, K_M must be expressed in terms of three rate constants. Nevertheless, K_M (in units of molarity) is customarily reported together with other kinetic parameters for enzyme-catalyzed reactions. To begin with, it is a quantity that can be measured easily and directly. Furthermore, K_M depends on temperature, the nature of the substrate, pH, ionic strength, and other reaction conditions; therefore, its value serves to characterize a particular enzyme–substrate system under specific conditions. Any variation in K_M (for the same enzyme and substrate) is often an indication of the presence of an inhibitor or activator. Useful information about evolution can also be obtained by comparing the K_M values of a similar enzyme from different species. For the majority of enzymes, K_M lies between 10^{-1} M and 10^{-7} M.

The maximum rate, V_{max}, has a well-defined meaning, both theoretically and empirically. It represents the maximum rate attainable; that is, it is the rate at which the total enzyme concentration is present as the enzyme–substrate complex. According to Equation 10.6, if $[E]_0$ is known, the value of k_2 can be determined from the value of V_{max} measured by one of the plots mentioned earlier. Note that k_2 is a first-order rate constant and has the unit of per unit time (s^{-1} or min^{-1}). It is called the *turnover number* (also referred to as k_{cat}, the *catalytic constant*). The turnover number of an enzyme is the number of substrate molecules (or moles of substrate) that are converted to product per unit time, when the enzyme is fully saturated with the substrate. For most enzymes, the turnover number varies between 1 and 10^5 s^{-1} under physiological conditions. Carbonic anhydrase, an enzyme that catalyzes the hydration of carbon dioxide and the dehydration of carbonic acid,

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

has one of the largest turnover numbers known ($k_2 = 1 \times 10^6$ s^{-1}) at 25°C. Thus, a 1×10^{-6} M solution of the enzyme can catalyze the formation of 1 M H_2CO_3 from CO_2 (produced by metabolism) and H_2O per second; that is,

$$\begin{aligned} V_{max} &= (1 \times 10^6 \text{ s}^{-1})(1 \times 10^{-6} \ M) \\ &= 1 \ M \text{ s}^{-1} \end{aligned}$$

Without the enzyme, the pseudo first-order rate constant is only about 0.03 s^{-1}. [Note that if the purity of the enzyme or the number of active sites per molecule is unknown, we cannot calculate the turnover number. In that case, the activity of the enzyme may be given as *units of activity per milligram of protein* (called the *specific activity*). One *international unit* is the amount of enzyme that produces one micromole (1 μmol) of product per minute.]

As stated, we can determine the turnover number by measuring the rate under saturating substrate conditions; that is, when $[S] \gg K_M$ (see Equation 10.8). Under physiological conditions, the ratio $[S]/K_M$ is seldom greater than one; in fact, it is frequently much smaller than one. When $[S] \ll K_M$, Equation 10.8 becomes

$$\begin{aligned} v_0 &= \frac{k_2}{K_M}[E]_0[S] \\ &= \frac{k_{cat}}{K_M}[E]_0[S] \end{aligned} \tag{10.13}$$

Note that Equation 10.13 expresses the rate law of a second-order reaction. It is interesting that the ratio k_{cat}/K_M (which has the units M^{-1} s^{-1}) is a measure of the

Table 10.1
Values of K_M, k_{cat}, and k_{cat}/K_M for Some Enzymes and Substrates

Enzyme	Substrate	K_M/M	k_{cat}/s^{-1}	$(k_{cat}/K_M)/M^{-1} \cdot s^{-1}$
Acetylcholin-esterase	Acetylcholine	9.5×10^{-5}	1.4×10^{4}	1.5×10^{8}
Catalase	H_2O_2	2.5×10^{-2}	1.0×10^{7}	4.0×10^{8}
Carbonic anhydrase	CO_2	0.012	1.0×10^{6}	8.3×10^{7}
Chymotrypsin	*N*-acetylglycine ethyl ester	0.44	5.1×10^{-2}	0.12
Fumarase	Fumarate	5.0×10^{-6}	8.0×10^{2}	1.6×10^{8}
Urease	Urea	2.5×10^{-2}	1.0×10^{4}	4.0×10^{5}

catalytic efficiency of an enzyme. A large ratio favors the formation of product. The reverse holds true for a small ratio.

Finally we ask the question: What is the upper limit of the catalytic efficiency of an enzyme? From Equation 10.9, we find

$$\frac{k_{cat}}{K_M} = \frac{k_2}{K_M} = \frac{k_1 k_2}{k_{-1} + k_2} \tag{10.14}$$

This ratio is a maximum when $k_2 \gg k_{-1}$; that is, k_1 is rate-determining and the enzyme turns over a product as soon as an ES complex is formed. However, k_1 can be no greater than the frequency of encounter between the enzyme and the substrate molecule, which is controlled by the rate of diffusion in solution.* The rate constant of a diffusion-controlled reaction is on the order of $10^8\ M^{-1}\ s^{-1}$. Therefore, enzymes with such k_{cat}/K_M values must catalyze a reaction almost every time they collide with a substrate molecule. Table 10.1 shows that acetylcholinesterase, catalase, fumarase, and perhaps carbonic anhydrase, have achieved this state of catalytic perfection.

10.3 Chymotrypsin: A Case Study

Having developed the basic equations of enzyme kinetics, we shall now consider some reactions catalyzed by chymotrypsin, a digestive enzyme. Aside from its important role in digestion, chymotrypsin catalysis is significant for being the system whose study provided the first evidence for the general existence of covalent enzyme–substrate complexes.

Chymotrypsin is one of the serine proteases, a family of protein-cutting enzymes that includes trypsin, elastase, and subtilisin. It has a molar mass of 24,800 daltons, 246 amino acid residues, and one active site (containing the serine residue) per molecule. Chymotrypsin is produced in the mammalian pancreas, where it takes the form of an inactive precursor, chymotrypsinogen. Once this precursor has entered the intestine, it is activated by another enzyme, trypsin, to become chymotrypsin. In this way, it avoids self-destruction before it can digest food. The enzyme can be prepared in highly purified form by crystallization.

* The rates of some enzyme-catalyzed reactions actually exceed the diffusion-controlled limit. When enzymes are associated with organized assemblies (for example, in cellular membranes), the product of one enzyme is channeled to the next enzyme, much as in an assembly line. In such cases, the rate of catalysis is not limited by the rate of diffusion in solution.

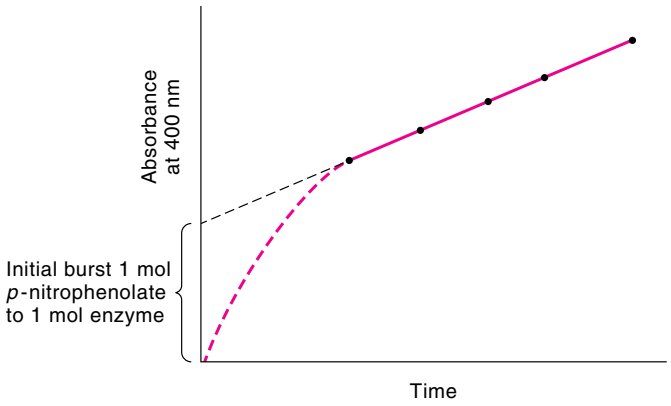
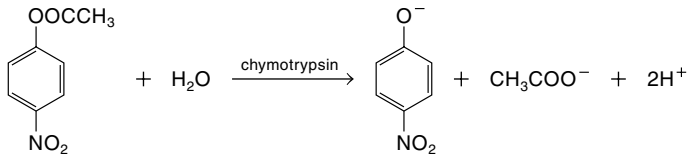


Figure 10.10
Chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate. The reaction shows an initial burst of *p*-nitrophenolate. Extrapolation of the absorbance to zero time shows 1 : 1 stoichiometry between the *p*-nitrophenolate produced and the amount of enzyme used.

In 1953, the British chemists B. S. Hartley and B. A. Kilby studied the hydrolysis of *p*-nitrophenyl acetate (PNPA), catalyzed by chymotrypsin to yield *p*-nitrophenolate ion and acetate ion:



This reaction can be monitored spectrophotometrically because *p*-nitrophenyl acetate is colorless, whereas *p*-nitrophenolate is bright yellow, with a maximum absorbance at 400 nm. Hartley and Kilby found that in the presence of a large excess of *p*-nitrophenyl acetate,* the release of *p*-nitrophenolate was linear with time. When they extrapolated the absorbance at 400 nm back to zero time, however, they found that it did not converge to zero absorbance (Figure 10.10). Kinetic measurements showed that the reaction proceeds with an initial burst of *p*-nitrophenolate release, followed by the usual zero-order release of *p*-nitrophenolate from turnover of the enzyme when it reaches the steady-state limit. The burst corresponds to one mole of *p*-nitrophenolate for each mole of enzyme, suggesting that the burst is the result of a chemical reaction between *p*-nitrophenyl acetate and chymotrypsin.

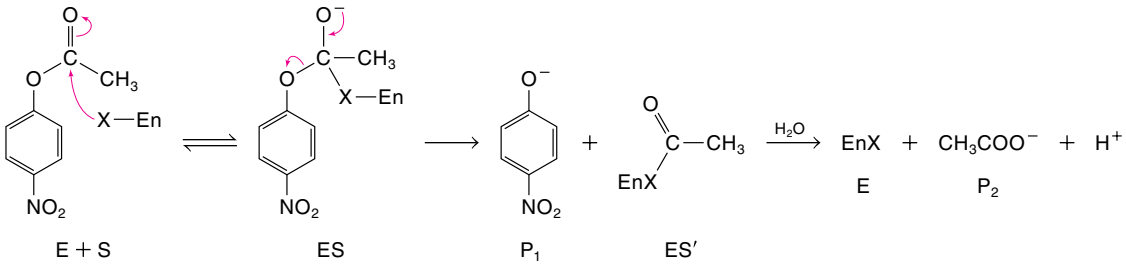
The chymotrypsin study clearly demonstrated that the reaction is *biphasic* (proceeds in two phases): the rapid reaction of the substrate with the enzyme, which yields a stoichiometric amount of *p*-nitrophenolate followed by a slower, steady-state reaction that produces the acetate ion. The following kinetic scheme is consistent with Hartley and Kilby's observations:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

where P_1 is *p*-nitrophenolate and P_2 is acetate. Furthermore, k_3 is the rate-

* A large excess of *p*-nitrophenyl acetate was used in the study because the enzyme has a very high K_M value.

determining step in the hydrolysis reaction. The reaction mechanism is



where X represents a nucleophilic group on the enzyme (En), which is the hydroxyl group of the serine residue at the active site. The first step is the rapid acylation of X by *p*-nitrophenol actetate, with the release of one equivalent mole of *p*-nitrophenolate in the burst.* Next is the slow hydrolysis of this acyl–enzyme intermediate (ES′), followed by the fast reacylation of the free enzyme by *p*-nitrophenol actetate, which accounts for the slow turnover of *p*-nitrophenolate production.

The chymotrypsin-catalyzed hydrolysis of *p*-nitrophenol acetate and related compounds is an example of *covalent hydrolysis*, a pathway in which part of the substrate forms a covalent bond with the enzyme to give an intermediate chemical species. In a second step, the intermediate undergoes another reaction to form the product and regenerate the free enzyme. The initial phase of the catalyzed reaction with *p*-nitrophenol acetate is so rapid that a stopped-flow apparatus must be employed to measure the progress of the reaction. However, the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate to *p*-nitrophenolate and trimethylacetate has the same characteristics as *p*-nitrophenyl acetate hydrolysis but proceeds much more slowly because the methyl groups constitute a steric barrier. Consequently, this reaction can be studied conveniently by means by a conventional spectrometer. Figure 10.11 shows a plot of the absorbance of *p*-nitrophenolate versus time with *p*-nitrophenyl trimethylacetate as the substrate.

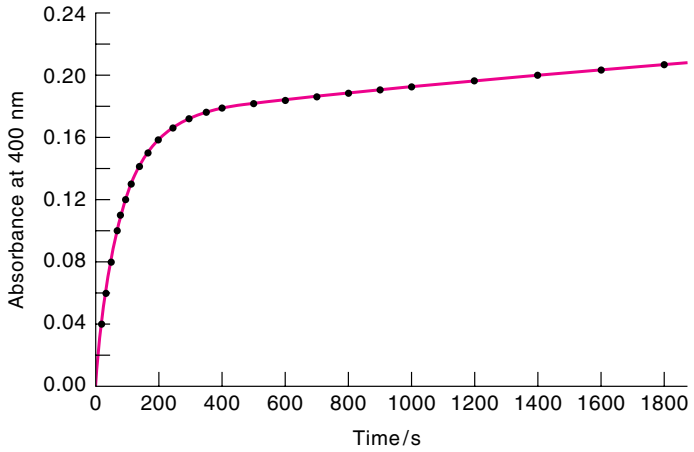


Figure 10.11
The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrotrimethylacetate at 298 K. [From M. L. Bender, F. J. Kézdy, and F. C. Wedler, *J. Chem. Educ.* **44**, 84 (1967).]

* In the formation of the ES complex, the proton from the hydroxyl group is transferred to a nearby histidine residue on chymotrypsin.

The kinetic analysis of this reaction—that is, the theoretical fit for the curve in Figure 10.10—starts with the following equations:

$$[E]_0 = [E] + [ES] + [ES']$$

$$\frac{d[P_1]}{dt} = k_2[ES]$$

$$\frac{d[P_2]}{dt} = k_3[ES']$$

$$\frac{d[ES']}{dt} = k_2[ES] - k_3[ES']$$

Because there are five unknowns ($k_2, k_3, [E]_0$, and two of the following three quantities: [E], [ES], and [ES′]) and only four equations, we need one more equation. For this equation, we assume that the first step is a rapid equilibrium; that is,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

and we write

$$K_S = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$

From these equations, we can fit the curve shown in Figure 10.10 and solve for the pertinent kinetic constants.* Table 10.2 shows the results. For this mechanism, the quantity k_{cat} (catalytic rate constant) is defined by

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{10.15}$$

For ester hydrolysis, $k_2 \gg k_3$, so k_{cat} is essentially equal to k_3.

Table 10.2
Kinetic Constants of the α-Chymotrypsin-Catalyzed Hydrolysis of *p*-Nitrophenyl Trimethylacetate at pH 8.2[a,b]

k_2	$0.37 \pm 0.11\ s^{-1}$
k_3	$(1.3 \pm 0.03) \times 10^{-4}\ s^{-1}$
K_s	$(1.6 \pm 0.5) \times 10^{-3}\ M$
k_{cat}	$1.3 \times 10^{-4}\ s^{-1}$
K_M	$5.6 \times 10^{-7}\ M^{-1}$

[a] From M. L. Bender, F. J. Kézdy, and F. C. Wedler, *J. Chem. Educ.* **44**, 84 (1967).
[b] 0.01 *M* tris–HCl buffer, ionic strength 0.06, 25.6 ± 0.1°C, 1.8% (v/v) acetonitrile–water.

10.4 Multisubstrate Systems

So far, we have considered enzyme catalysis involving only a single substrate, but in many cases, the process involves two or more substrates. For example, the reaction

$$C_2H_5OH + NAD^+ \rightleftharpoons CH_3CHO + NADH + H^+$$

is catalyzed by the enzyme alcohol dehydrogenase, which binds both NAD^+ and the substrate that is to be oxidized. Many of the principles developed for a single-substrate system may be extended to multisubstrate systems. Ignoring mathematical details, we shall briefly examine the different types of bisubstrate reactions—that is, reactions involving two substrates.

The overall picture of a bisubstrate reaction can be represented by

$$A + B \rightleftharpoons P + Q$$

where A and B are the substrates and P and Q the products. In most cases, these

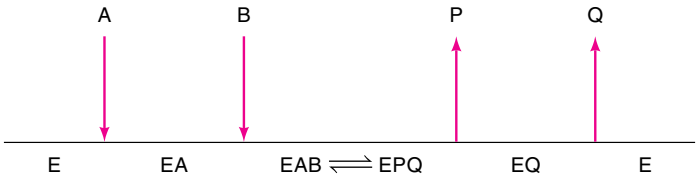
* For the derivation, see Reference 3 on p. 6.

reactions involve the transfer of a specific functional group from one substrate (A) to the other (B). The binding of A and B to the enzyme can take place in different ways, which can be categorized as sequential or nonsequential mechanisms.

The Sequential Mechanism

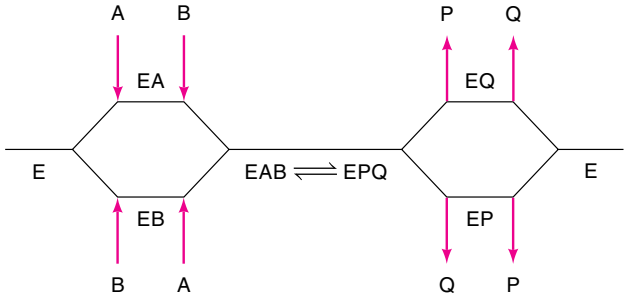
In some reactions, the binding of both substrates must take place before the release of products. A sequential process can be further classified as follows.

Ordered Sequential Mechanism. In this mechanism, one substrate must bind before a second substrate can bind.



The enzyme and enzyme–substrate complexes are represented by horizontal lines, and successive additions of substrates and release of products are denoted by vertical arrows. Each vertical arrow actually represents the forward and reverse reaction. This mechanism is often observed in the oxidation of substrates by NAD^+.

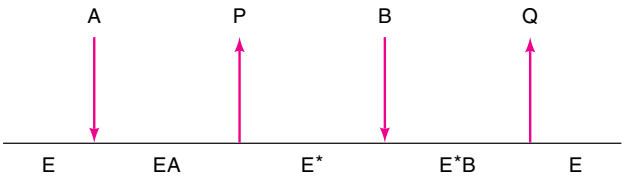
Random Sequential Mechanism. The case in which the binding of substrates and the release of products do not follow a definite obligatory order is known as a random sequential mechanism. The general pathway is as follows:



The phosphorylation of glucose by ATP to form glucose-6-phosphate, in which hexokinase is the enzyme in the first step of glycolysis, appears to follow such a mechanism.

The Nonsequential or "Ping-Pong" Mechanism

In this mechanism, one substrate binds, and one product is released. Then, a second substrate binds, and a second product is released.



This process is called the "Ping-Pong mechanism" to emphasize the bouncing of the enzyme between the two states E and E^*, where E^* is a modified state of E, which often carries a fragment of A. An example of the Ping-Pong mechanism is the action by chymotrypsin (discussed on p. 372).

10.5 Enzyme Inhibition

Inhibitors are compounds that decrease the rate of an enzyme-catalyzed reaction. The study of enzyme inhibition has enhanced our knowledge of specificity and the nature of functional groups at the active site. The activity of certain enzymes is regulated by a feedback mechanism such that an end product inhibits the enzyme's function in an initial stage of a sequence of reactions (Figure 10.12). The glycolytic pathway is

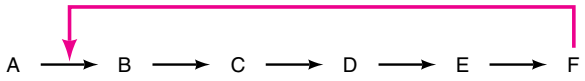


Figure 10.12
Control of regulatory enzymes frequently involves feedback mechanisms. In this sequence of reactions catalyzed by enzymes, the first enzyme in the series is inhibited by product F. At the early stages of the reaction, the concentration of F is low and its inhibitory effect is minimal. As the concentration of F reaches a certain level, it can lead to total inhibition of the first enzyme and hence turns off its own source of production. This action is analogous to a thermostat turning off heat supply when the ambient temperature reaches a preset level.

an example of this feedback mechanism. In effect, enzyme inhibition controls the amount of products formed.

The action of an inhibitor on an enzyme can be described as either reversible or irreversible. In *reversible inhibition*, an equilibrium exists between the enzyme and the inhibitor. In *irreversible inhibitions*, inhibition progressively increases with time. Complete inhibition results if the concentration of the irreversible inhibitor exceeds that of the enzyme.

Reversible Inhibition

There are three important types of reversible inhibition: competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition. We shall discuss each type in turn.

Competitive Inhibition. In this case, both the substrate S and the inhibitor I compete for the same active site (Figure 10.13a). The reactions are

$$\begin{array}{ccccccccc} \mathrm{E} & + & \mathrm{S} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{ES} & \xrightarrow{k_2} & \mathrm{P} & + & \mathrm{E} \\ + & & & & & & & & \\ \mathrm{I} & & & & & & & & \\ \Big\Updownarrow K_\mathrm{I} & & & & & & & & \\ \mathrm{EI} & & & & & & & & \end{array}$$

where

$$K_\mathrm{I} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} \tag{10.16}$$

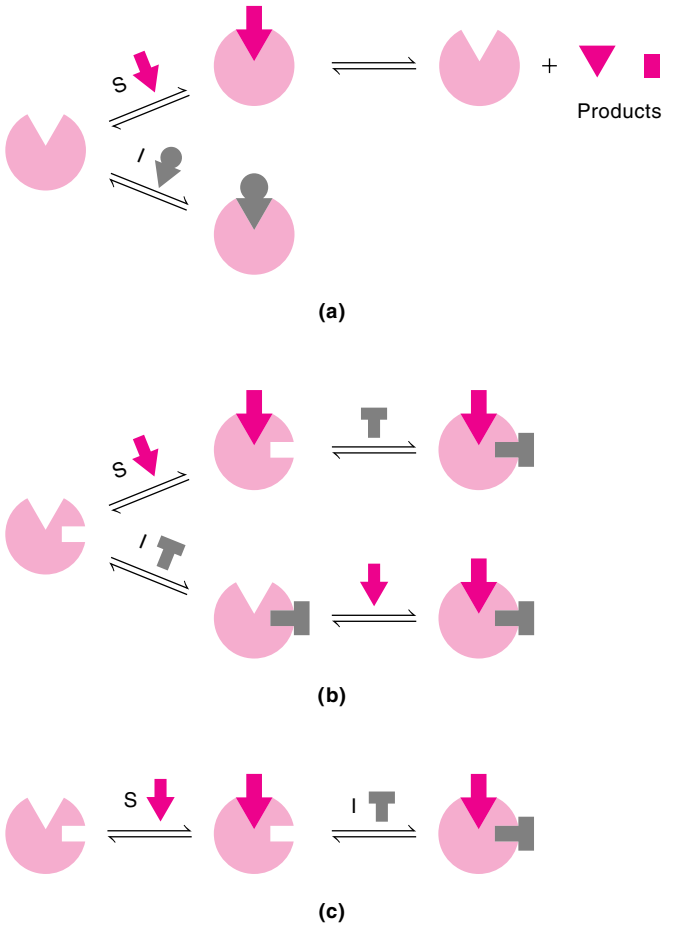


Figure 10.13
Three types of reversible inhibition. (a) Competitive inhibition. Both the substrate and the inhibitor compete for the same active site. Only the ES complex leads to production formation. (b) Noncompetitive inhibition. The inhibitor binds to a site other than the active site. The ESI complex does not lead to product formation. (c) Uncompetitive inhibition. The inhibitor binds only to the ES complex. The ESI complex does not lead to product formation.

Note that the complex EI does not react with S to form products. Applying the steady-state approximation for ES, we obtain*

$$v_0 = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{M}}\left(1 + \dfrac{[\mathrm{I}]}{K_{\mathrm{I}}}\right) + [\mathrm{S}]} \tag{10.17}$$

Equation 10.17 has the same form as Equation 10.10, except that the K_{M} term has been modified by $(1 + [\mathrm{I}]/K_{\mathrm{I}})$, thereby reducing v_0. The Lineweaver–Burk equation is given by

$$\frac{1}{v_0} = \frac{K_{\mathrm{M}}}{V_{\max}}\left(1 + \frac{[\mathrm{I}]}{K_{\mathrm{I}}}\right)\frac{1}{[\mathrm{S}]} + \frac{1}{V_{\max}} \tag{10.18}$$

* For derivations of Equations 10.17 and 10.19, see Reference 3 on p. 6.

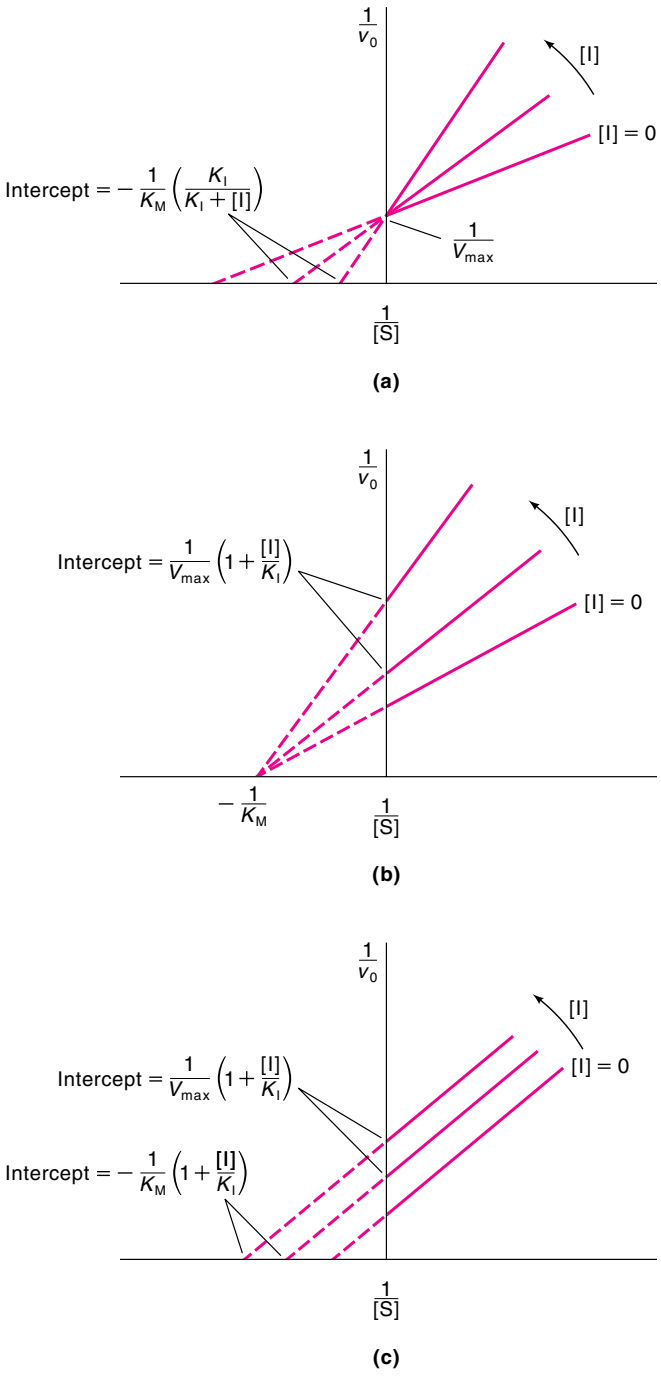
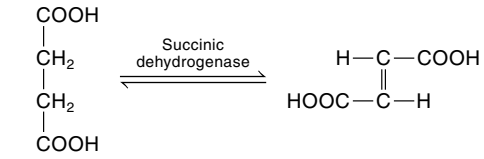


Figure 10.14
Lineweaver–Burk plots: (a) competitive inhibition, (b) noncompetitive inhibition, and (c) uncompetitive inhibition.

Thus, a straight line results when $1/v_0$ is plotted versus $1/[S]$ at constant [I] (Figure 10.14a). The difference between Equations 10.18 and 10.11 is that in the former, the slope is enhanced by the factor $(1 + [I]/K_I)$. The intercept on the $1/v_0$ axis is the same for Figures 10.13a and 10.8 because V_{max} does not change.

A well-known example of a competitive inhibitor is malonic acid, $CH_2(COOH)_2$, which competes with succinic acid in the dehydrogenation reaction catalyzed by

succinic dehydrogenase:



Succinic acid Fumaric acid

Because malonic acid resembles succinic acid in structure, it can combine with the enzyme, although no product is formed in this reaction.

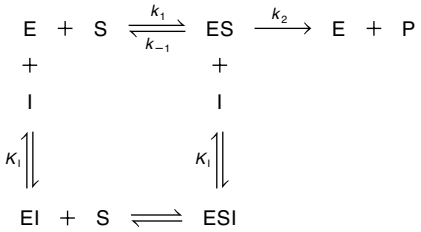
Dividing Equation 10.10 by Equation 10.17, we obtain

$$\frac{v_0}{(v_0)_{\text{inhibition}}} = 1 + \frac{K_M[I]}{K_M K_I + [S]K_I}$$

To overcome competitive inhibition, we need to increase the substrate concentration relative to that of the inhibitor; that is, at high substrate concentrations, $[S]K_I \gg K_M K_I$, so that

$$\frac{v_0}{(v_0)_{\text{inhibition}}} \approx 1 + \frac{K_M[I]}{[S]K_I} \approx 1$$

Noncompetitive Inhibition. A noncompetitive inhibitor binds to the enzyme at a site that is distinct from the substrate binding site; therefore, it can bind to both the free enzyme and the enzyme–substrate complex (see Figure 10.13b). The binding of the inhibitor has no effect on the substrate binding, and vice versa. The reactions are



Neither EI nor ESI forms products. Because I does not interfere with the formation of ES, noncompetitive inhibition cannot be reversed by increasing the substrate concentration. The initial rate is given by

$$v_0 = \frac{\dfrac{V_{\max}}{\left(1 + \dfrac{[I]}{K_I}\right)}[S]}{K_M + [S]} \tag{10.19}$$

Comparing Equation 10.19 with Equation 10.10, we see that $V_{\max}$ has been reduced by the factor $(1 + [I]/K_I)$ but K_M is unchanged. The Lineweaver–Burk equation becomes

$$\frac{1}{v_0} = \frac{K_M}{V_{\max}}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V_{\max}}\left(1 + \frac{[I]}{K_I}\right) \tag{10.20}$$

From Figure 10.14b we see that a plot of $1/v_0$ versus $1/[S]$ gives a straight line with an increase in slope and intercept on the $1/v_0$ axis compared with that in Figure 10.8. Dividing Equation 10.10 by Equation 10.19, we get

$$\frac{v_0}{(v_0)_{\text{inhibition}}} = 1 + \frac{[I]}{K_I}$$

This result confirms our earlier statement that the extent of noncompetitive inhibition is independent of [S] and depends only on [I] and K_I.

Noncompetitive inhibition is very common with multisubstrate enzymes. Other examples are the reversible reactions between the sulfhydryl groups of cysteine residues on enzymes with heavy metal ions:

$$2\text{–SH} + Hg^{2+} \rightleftharpoons \text{–S–Hg–S–} + 2H^+$$
$$\text{–SH} + Ag^+ \rightleftharpoons \text{–S–Ag} + H^+$$

Uncompetitive Inhibition. An uncompetitive inhibitor does not bind to the free enzyme; instead, it binds reversibly to the enzyme–substrate complex to yield an inactive ESI complex (see Figure 10.13c). The reactions are

$$\begin{array}{ccccccc} E & + & S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & E + P \\ & & & & + & & \\ & & & & I & & \\ & & & & \Big\uparrow\Big\downarrow K_I & & \\ & & & & ESI & & \end{array}$$

where

$$K_I = \frac{[ES][I]}{[ESI]} \tag{10.21}$$

The ESI complex does not form a product. Again, because I does not interfere with the formation of ES, uncompetitive inhibition cannot be reversed by increasing the substrate concentration. The initial rate is given by (see Problem 10.16)

$$v_0 = \frac{\dfrac{V_{\max}}{\left(1 + \dfrac{[I]}{K_I}\right)}[S]}{\dfrac{K_M}{\left(1 + \dfrac{[I]}{K_I}\right)} + [S]} \tag{10.22}$$

Comparison of Equation 10.22 with Equation 10.10 shows that both $V_{\max}$ and K_M have been reduced by the factor $(1 + [I]/K_I)$. The Lineweaver–Burk equation is given by

$$\frac{1}{v_0} = \frac{K_M}{V_{\max}}\frac{1}{[S]} + \frac{1}{V_{\max}}\left(1 + \frac{[I]}{K_I}\right) \tag{10.23}$$

Thus, a straight line is obtained by plotting $1/v_0$ versus $1/[S]$ at constant [I] (see Figure 10.14c). The difference between Equation 10.23 and 10.11 is that the intercept

on the $1/v_0$ axis is altered by the factor $(1 + [\mathrm{I}]/K_\mathrm{I})$, but the slope remains the same. Dividing Equation 10.10 by Equation 10.22, we get

$$\frac{v_0}{(v_0)_{\text{inhibition}}} = \frac{K_\mathrm{M} + [\mathrm{S}](1 + [\mathrm{I}]/K_\mathrm{I})}{K_\mathrm{M} + [\mathrm{S}]}$$

If conditions are such that $[\mathrm{S}] \gg K_\mathrm{M}$, then the equation above becomes

$$\frac{v_0}{(v_0)_{\text{inhibition}}} = \frac{[\mathrm{S}] + [\mathrm{S}][\mathrm{I}]/K_\mathrm{I}}{[\mathrm{S}]} = 1 + \frac{[\mathrm{I}]}{K_\mathrm{I}}$$

Again we see that increasing the substrate concentration cannot overcome the effect of I in uncompetitive inhibition, just as in the case of noncompetitive inhibition.

Uncompetitive inhibition is rarely observed in one-substrate systems. Multisubstrate enzymes, however, often give parallel line plots with inhibitors.

Example 10.1

A chemist measured the initial rate of an enzyme-catalyzed reaction in the absence and presence of inhibitor A and, in a separate procedure, inhibitor B. In each case, the inhibitor's concentration was 8.0 mM (8.0×10^{-3} M). The following data were obtained:

$[\mathrm{S}]/M$	$v_0/M \cdot \mathrm{s}^{-1}$ No Inhibitor	$v_0/M \cdot \mathrm{s}^{-1}$ Inhibitor A	$v_0/M \cdot \mathrm{s}^{-1}$ Inhibitor B
5.0×10^{-4}	1.25×10^{-6}	5.8×10^{-7}	3.8×10^{-7}
1.0×10^{-3}	2.0×10^{-6}	1.04×10^{-6}	6.3×10^{-7}
2.5×10^{-3}	3.13×10^{-6}	2.00×10^{-6}	1.00×10^{-6}
5.0×10^{-3}	3.85×10^{-6}	2.78×10^{-6}	1.25×10^{-6}
1.0×10^{-2}	4.55×10^{-6}	3.57×10^{-6}	1.43×10^{-6}

(a) Determine the values of K_M and $V_{\max}$ of the enzyme. (b) Determine the type of inhibition imposed by inhibitors A and B, and calculate the value of K_I in each case.

ANSWER

Our first step is to convert the data to $1/[\mathrm{S}]$ and $1/v_0$:

$(1/[\mathrm{S}])/M^{-1}$	$(1/v_0)/M^{-1} \cdot \mathrm{s}$ No Inhibitor	$(1/v_0)/M^{-1} \cdot \mathrm{s}$ Inhibitor A	$(1/v_0)/M^{-1} \cdot \mathrm{s}$ Inhibitor B
2.0×10^3	8.0×10^5	1.72×10^6	2.63×10^6
1.0×10^3	5.0×10^5	9.6×10^5	1.60×10^6
4.0×10^2	3.2×10^5	5.0×10^5	1.00×10^5
2.0×10^2	2.6×10^5	3.6×10^5	8.0×10^5
1.0×10^2	2.2×10^5	2.8×10^5	7.0×10^5

Next, we draw the Lineweaver–Burk plots for these three sets of kinetic data, as shown in Figure 10.15. Comparing Figure 10.15 with Figures 10.14a, 10.14b, and 10.14c shows that A is a competitive inhibitor and B is a noncompetitive inhibitor.

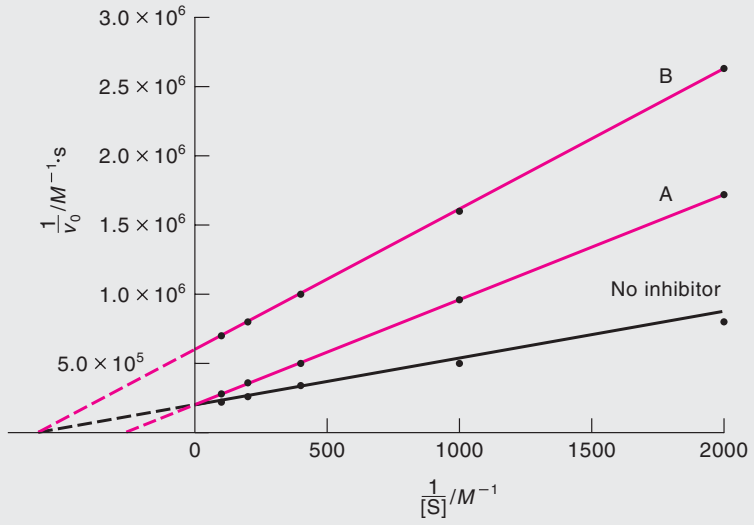


Figure 10.15
Lineweaver–Burk plots to determine the kinetic parameters and types of inhibition for Example 10.1.

(a) The computer linear fit for no inhibition is

$$\frac{1}{v_0} = 302.6\frac{1}{[\mathrm{S}]} + 1.96 \times 10^5$$

From Equation 10.11 we find that

$$\frac{1}{V_{max}} = 1.96 \times 10^5\ M^{-1}\ \mathrm{s}$$

or

$$V_{max} = 5.1 \times 10^{-6}\ M\ \mathrm{s}^{-1}$$

From the slope of the line,

$$302.6\ \mathrm{s} = \frac{K_\mathrm{M}}{V_{max}}$$

so

$$\begin{aligned} K_\mathrm{M} &= (302.6\ \mathrm{s})(5.1 \times 10^{-6}\ M\ \mathrm{s}^{-1}) \\ &= 1.5 \times 10^{-3}\ M \end{aligned}$$

(b) The computer linear fit for inhibitor A is

$$\frac{1}{v_0} = 757.8\frac{1}{[\mathrm{S}]} + 2.03 \times 10^5$$

[Note that the slight difference in the $1/V_{max}$ value (2.03×10^5) compared with 1.96×10^5 for the no-inhibition plot is due to experimental uncertainty.] From Equation

10.18 the slope is equated as follows:

$$757.8 \text{ s} = \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_I}\right)$$
$$= \frac{1.5 \times 10^{-3}\ M}{5.1 \times 10^{-6}\ M\ \text{s}^{-1}}\left(1 + \frac{[I]}{K_I}\right)$$

Because $[I] = 8.0 \times 10^{-3}\ M$,

$$K_I = 5.1 \times 10^{-3}\ M$$

The computer linear fit for inhibitor B is

$$\frac{1}{v_0} = 1015.3\frac{1}{[S]} + 5.95 \times 10^5$$

From Equation 10.20, we express the slope as

$$1015.3 \text{ s} = \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_I}\right)$$
$$= \frac{1.5 \times 10^{-3}\ M}{5.1 \times 10^{-6}\ M\ \text{s}^{-1}}\left(1 + \frac{[I]}{K_I}\right)$$

Because $[I] = 8.0 \times 10^{-3}\ M$,

$$K_I = 3.3 \times 10^{-3}\ M$$

Irreversible Inhibition

Michaelis–Menten kinetics cannot be applied to irreversible inhibition. The inhibitor forms a covalent linkage with the enzyme molecule and cannot be removed. The effectiveness of an irreversible inhibitor is determined not by the equilibrium constant but by the rate at which the binding takes place. Iodoacetamides and maleimides act as irreversible inhibitors to the sulfhydryl groups:

$$-SH + ICH_2CONH_2 \rightarrow -S-CH_2CONH_2 + HI$$

Another example is the action of diisopropyl phosphofluoridate (a nerve gas) on the enzyme acetylcholinesterase. When a nerve makes a muscle cell contract, it gives the cell a tiny squirt of acetylcholine molecules. Acetylcholine is called a neurotransmitter because it acts as a messenger between the nerve and the final destination (in this case, the muscle cell). Once they have performed the proper function, the acetylcholine molecules must be destroyed; otherwise, the resulting excess of this substance will hyperstimulate glands and muscle, producing convulsions, choking, and other distressing symptoms. Many victims of exposure to this nerve gas suffer paralysis or even death. The effective removal of excess acetylcholine is by means of a hydrolysis reaction (see Section 7.5):

$$\underset{\text{Acetylcholine}}{CH_3COOCH_2CH_2-\overset{+}{N}(CH_3)_3} + H_2O \rightarrow \underset{\text{Choline}}{HOCH_2CH_2-\overset{+}{N}(CH_3)_3} + CH_3COOH$$

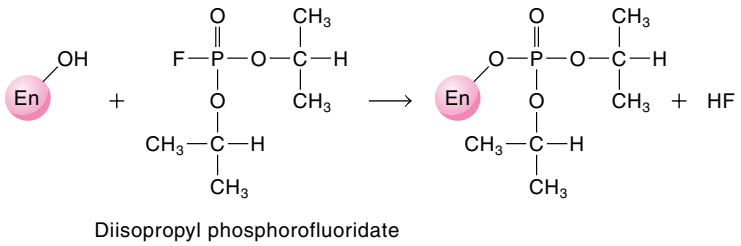


Figure 10.16
An example of irreversible inhibition. The nerve gas diisopropyl phosphorofluoridate forms a strong covalent bond with the hydroxyl group of the serine residue at the active site of acetylcholinesterase.

The catalyst for this reaction is acetylcholinesterase. The irreversible inhibition of this enzyme takes place via the formation of a covalent bond between the phosphorus atom and the hydroxyl oxygen of the serine residue in the enzyme (Figure 10.16). The complex formed is so stable that for practical purposes the restoration of normal nerve function must await the formation of new enzyme molecules by the exposed person's body.

10.6 Allosteric Interactions

One class of enzymes has kinetics that do not obey the Michaelis–Menten description. Instead of the usual hyperbolic curve (see Figure 10.5), the rate equations of these enzymes produce a sigmoidal, or S-shaped, curve. This behavior is typically exhibited by enzymes that possess multiple binding sites and whose activity is regulated by the binding of inhibitors or activators. Sigmoidal curves are characteristic of positive cooperativity, which means that the binding of the ligand at one site increases the enzyme's affinity for another ligand at a different site. Enzymes that show cooperativity are called *allosteric* (from the Greek words *allos*, meaning different, and *steros*, meaning space or solid, which means conformation in our discussion). The term *effector* describes the ligand that can affect the binding at a different site on the enzyme. There are four types of allosteric interactions, depending on whether the ligands are of the same type (*homotropic effect*) or different type (*heterotropic effect*): positive or negative homotropic effect and positive or negative heterotropic effect. The words *positive* and *negative* here describe the enzyme's affinity for other ligands as a result of the binding to the effector.

Oxygen Binding to Myoglobin and Hemoglobin

Hemoglobin is sometimes referred to as the honorary enzyme.

The phenomenon of cooperativity was first observed for the oxygen–hemoglobin system. Although hemoglobin is not an enzyme, its mode of binding with oxygen is analogous to binding by allosteric enzymes. Figure 10.17 shows the percent saturation curves for hemoglobin and myoglobin. A hemoglobin molecule is made up of four polypeptide chains, two α chains of 141 amino acid residues each and two β chains of 146 amino acid residues each. Each chain contains a heme group. The iron atom in the heme group has octahedral geometry; it is bonded to the four nitrogen atoms of the heme group and the nitrogen atom of the histidine residue, leaving a sixth coordination site open for ligand binding (water or molecular oxygen). The four chains fold to form similar three-dimensional structures. In an intact hemoglobin molecule, these four chains, or *subunits*, are joined together to form a tetramer. The

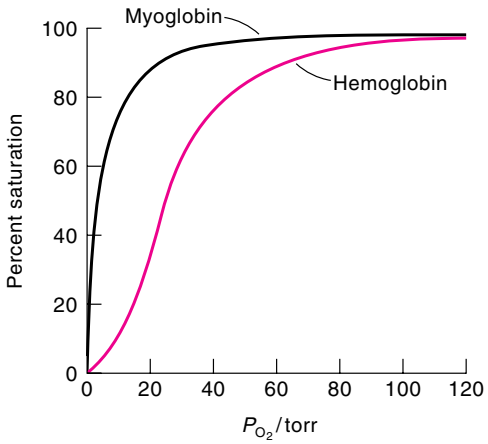
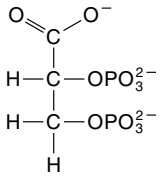


Figure 10.17
Oxygen saturation curves for myoglobin and hemoglobin.

less complex myoglobin molecule possesses only one polypeptide of 153 amino acids. It contains one heme group and is structurally similar to the β chain of hemoglobin. As we can see in Figure 10.17, the curve for myoglobin is hyperbolic, indicating that it binds noncooperatively with oxygen. This observation is consistent with the fact that there is only one heme group and hence only one binding site. On the other hand, the curve for hemoglobin is sigmoidal, indicating that its affinity for oxygen increases with the binding of oxygen.

Because of the great physiological significance of the binding of oxygen to hemoglobin, we need to look at the process in more detail. The oxygen affinity for hemoglobin depends on the concentration of several species in the red blood cell: protons, carbon dioxide, chloride ions, and 2,3-bisphosphoglycerate (BPG) [once known as 2,3-diphosphoglycerate (DPG)],



An increase in the concentration of any of these species shifts the oxygen binding curve (see Figure 10.17) to the right, which indicates a decrease in the oxygen affinity. Thus, all of these ligands act as negative heterotropic effectors. In the tissues, where the partial pressure of carbon dioxide and the concentration of H^+ ions are high, the oxyhemoglobin molecules have a greater tendency to dissociate into hemoglobin and oxygen, and the latter is taken up by myoglobin for metabolic processes. About two protons are taken up by the hemoglobin molecule for every four oxygen molecules released. The reverse effect occurs in the alveolar capillaries of the lungs. The high concentration of oxygen in the lungs drives off protons and carbon dioxide bound to deoxyhemoglobin. This reciprocal action, known as the *Bohr effect*, was first reported by the Danish physiologist Christian Bohr (1855–1911) in 1904. Figure 10.18a shows the effect of pH on the oxygen affinity of hemoglobin.

The effect of BPG on the oxygen affinity for hemoglobin was discovered by the American biochemists Reinhold Benesch (1919–1986) and Ruth Benesch (1925–) in 1967. They found that BPG binds only to deoxyhemoglobin and not to oxyhemoglobin, and that BPG reduces the oxygen affinity by a factor of about 25 (Figure 10.18b). The number of BPG molecules in the red blood cell is roughly the same as the number of hemoglobin molecules (280 million), but a shortage of oxygen triggers an increase in BPG, which promotes the release of oxygen. Interestingly, when

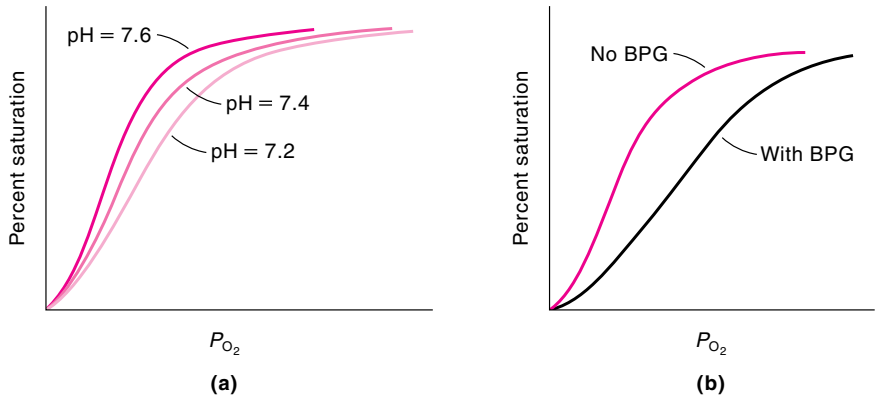


Figure 10.18
(a) The Bohr effect. A decrease in pH leads to a lowering of oxygen affinity for hemoglobin. (b) The presence of BPG decreases the oxygen affinity for hemoglobin.

a person travels quickly from sea level to a high-altitude region, where the partial pressure of oxygen is low, the level of BPG in his or her red blood cells increases. This increase lowers the oxygen affinity of hemoglobin and helps maintain a higher concentration of free oxygen. The human fetus has its own kind of hemoglobin, called hemoglobin F, which consists of two α chains and two γ chains. This hemoglobin differs from adult hemoglobin A, which consists of two α chains and two β chains. Under normal physiological conditions, hemoglobin F has a higher oxygen affinity than hemoglobin A. This difference in affinity promotes the transfer of oxygen from the maternal to the fetal circulation. The higher oxygen affinity for hemoglobin F is due to the fact that this molecule binds BPG less strongly than hemoglobin A. The comparison of these systems also shows that BPG binds only to the β chains in hemoglobin A and only to the γ chains in hemoglobin F.

Finally, the binding of oxygen to myoglobin is not affected by any of these factors. It does vary, however, with temperature. The oxygen affinity of both myoglobin and hemoglobin decreases with increasing temperature.

The Hill Equation

We now present a phenomenological description for the binding of oxygen to myoglobin and hemoglobin. Consider, first, the binding of oxygen to myoglobin (Mb), because it is a simpler system. The reaction is

$$\mathrm{Mb} + \mathrm{O_2} \rightleftharpoons \mathrm{MbO_2}$$

The dissociation constant is given by

$$K_\mathrm{d} = \frac{[\mathrm{Mb}][\mathrm{O_2}]}{[\mathrm{MbO_2}]} \tag{10.24}$$

We define a quantity Y, the fractional saturation, as follows (see p. 210):

$$Y = \frac{[\mathrm{MbO_2}]}{[\mathrm{MbO_2}] + [\mathrm{Mb}]} \tag{10.25}$$

From Equation 10.24 and 10.25,

$$Y = \frac{[O_2]}{[O_2] + K_d} \tag{10.26}$$

Because O_2 is a gas, expressing its concentration in terms of its partial pressure is more convenient. Furthermore, if we represent the oxygen affinity for myoglobin as P_{50}, which is the partial pressure of oxygen when half, or 50%, of the binding sites are filled (that is, when $[Mb] = [MbO_2]$), it follows that

$$K_d = \frac{[Mb]P_{O_2}}{[MbO_2]} = P_{O_2} = P_{50}$$

and Equation 10.26 becomes

$$Y = \frac{P_{O_2}}{P_{O_2} + P_{50}} \tag{10.27}$$

Rearranging, we have

$$\frac{Y}{1 - Y} = \frac{P_{O_2}}{P_{50}}$$

Taking the logarithm of both sides of the above equation, we obtain

$$\log \frac{Y}{1 - Y} = \log P_{O_2} - \log P_{50} \tag{10.28}$$

Thus, a plot of $\log(Y/1 - Y)$ versus $\log P_{O_2}$ gives a straight line with a slope of unity (Figure 10.19).

Equation 10.28 describes the binding of myoglobin with oxygen quite well, but it does not hold for hemoglobin. Instead, it must be modified as follows:

$$\log \frac{Y}{1 - Y} = n \log P_{O_2} - n \log P_{50} \tag{10.29}$$

A similar plot in this case yields a straight line with a slope of 2.8 (that is, $n = 2.8$), also shown in Figure 10.19. The fact that the slope is greater than unity indicates that the binding of hemoglobin with oxygen is cooperative. Note that we cannot explain

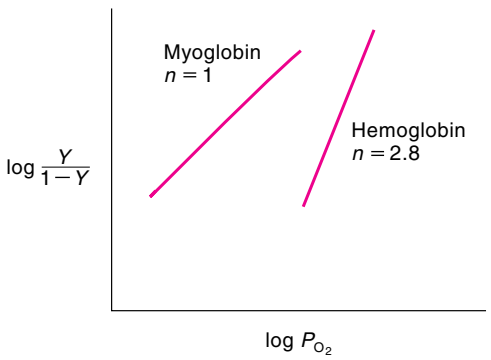


Figure 10.19
Plots of $\log(Y/1 - Y)$ versus $\log P_{O_2}$ for hemoglobin and myoglobin.

the binding phenomenon in this case by assuming that it is a higher-order reaction, because n is not an integer and it is not identical to the number of sites. (If all four sites were equivalent and independent of one another, we would analyze the binding curve using Equation 6.30.) Furthermore, the fact that the slope of the line at very low and very high partial pressures of oxygen tends to unity is inconsistent with a high-order mechanism, which predicts a constant slope at all partial pressures of oxygen. Equation 10.29 is often referred to as the *Hill equation* (after the British biochemist Archibald Vivian Hill, 1886–1977), and n is known as the *Hill coefficient*. The Hill coefficient is a measure of cooperativity—the higher n is, the higher the cooperativity. If $n = 1$, there is no cooperativity; if $n < 1$, there is negative cooperativity. The upper limit of n is the number of binding sites, which is 4 for hemoglobin.

What is the significance of cooperativity? In essence, it enables hemoglobin to be a more efficient oxygen transporter than myoglobin. The partial pressure of oxygen is about 100 torr in the lungs, compared with about 20 torr in the capillaries of muscle. Furthermore, the partial pressure for 50% saturation of hemoglobin is about 26 torr (see Figure 10.17). From Equation 10.29,

$$\frac{Y}{1 - Y} = \left(\frac{P_{O_2}}{P_{50}}\right)^n$$

In the lungs,

$$\frac{Y_{\text{lung}}}{1 - Y_{\text{lung}}} = \left(\frac{100}{26}\right)^{2.8}$$

or

$$Y_{\text{lung}} = 0.98$$

In the muscles,

$$\frac{Y_{\text{muscle}}}{1 - Y_{\text{muscle}}} = \left(\frac{20}{26}\right)^{2.8}$$

$$Y_{\text{muscle}} = 0.32$$

The amount of oxygen delivered is proportional to ΔY, given by

$$\Delta Y = Y_{\text{lung}} - Y_{\text{muscle}} = 0.66$$

What would happen if the binding between hemoglobin and oxygen were not cooperative? In this case, $n = 1$ and we have, from Equation 10.27,

$$Y_{\text{lung}} = \frac{100}{100 + 26} = 0.79$$

In the muscles,

$$Y_{\text{muscle}} = \frac{20}{20 + 26} = 0.43$$

so that $\Delta Y = 0.36$. Thus, almost twice as much oxygen is delivered to the tissues when the binding of hemoglobin with oxygen is cooperative.

Equation 10.29 is an empirical approach to cooperativity; it says nothing about the mechanism involved. Over the past 60 years, several theories have been proposed to explain cooperativity. Next, we shall briefly discuss two theories that have played important roles in our understanding of allosteric interactions.

The Concerted Model

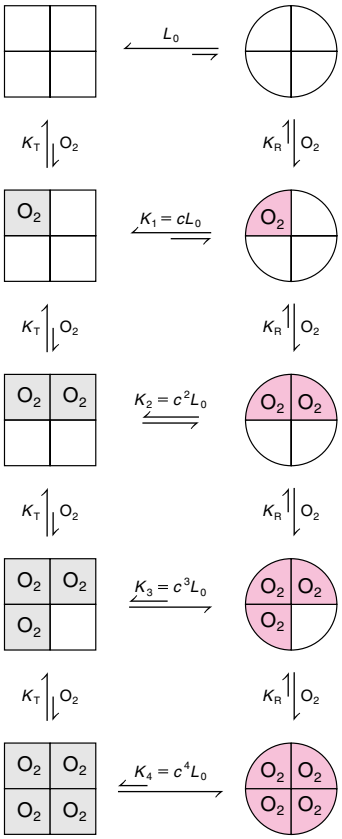


Figure 10.20
The concerted model for binding of oxygen with hemoglobin. The squares represent the tense state; the quarter circles represent the relaxed state.

In 1965, Monod, Wyman, and Changeux proposed a theory, called the *concerted model*, to explain cooperativity.* Their theory makes the following assumptions: (1) Proteins are oligomers; that is, they contain two or more subunits. (2) Each protein molecule can exist in either of two states, called T (tense) and R (relaxed), which are in equilibrium. (3) In the absence of substrate molecules, the T state is favored. When substrate molecules are bound to the enzyme, the equilibrium gradually shifts to the R state, which has a higher affinity for the ligand. (4) All binding sites in each state are equivalent and have an identical dissociation constant for the binding of ligands (K_T for the T state and K_R for the R state). Figure 10.20 shows the concerted model for the binding of oxygen with hemoglobin.

The equilibrium constant, L_0, for the two states in the absence of oxygen (denoted by the subscript 0) is given by

$$L_0 = \frac{[T_0]}{[R_0]} \tag{10.30}$$

Because the T state is favored in the absence of O_2, L_0 is large, and only negligible amounts of the R state are present. When oxygen is present, the equilibrium shifts gradually to the R state, which has a higher affinity for oxygen. When four molecules of ligand are bound, virtually all of the hemoglobin molecules will be in the R state, which corresponds to the conformation of oxyhemoglobin. We define c as the ratio of the dissociation constants:

$$c = \frac{K_R}{K_T} \tag{10.31}$$

Because the R state has the higher affinity for O_2, c must be smaller than 1. The affinity of a subunit for the ligand depends solely on whether it is in the T or R state and not on whether the sites on neighboring units are occupied; thus, K_R and K_T are the same for all stages of saturation. When one ligand is bound, the [T]/[R] ratio changes by the factor c; when two ligands are bound, the [T]/[R] ratio changes by c^2, and so on. We can represent the successive ligand binding depicted in Figure 10.20 with the following equations (see Problem 10.28):

$$K_1 = cL_0$$
$$K_2 = cK_1 = c^2L_0$$
$$K_3 = cK_2 = c^3L_0$$
$$K_4 = cK_3 = c^4L_0$$

We see that the equilibrium between T and R shifts to the R form as more O_2 molecules are bound.

Note that if hemoglobin were always entirely in the T state, its binding of oxygen, although weak, would be completely noncooperative and characterized only by

* J. Monod, J. Wyman, and P. P. Changeaux, *J. Mol. Biol.* **12**, 88 (1965).

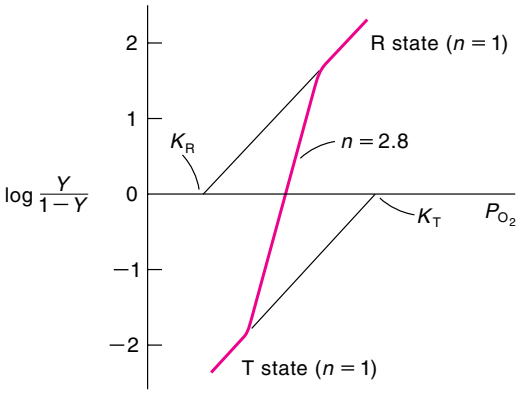


Figure 10.21
Plot of $\log(Y/1 - Y)$ versus P_{O_2} for hemoglobin.

K_T. Conversely, if hemoglobin were always entirely in the R state, its binding of oxygen, although strong, would also be completely noncooperative and characterized only by K_R. This noncooperativity is attributable to the fact that in any given hemoglobin molecule, all four subunits must either be in the R state (R_4) or the T state (T_4). Mixed forms, such as R_3T or R_2T_2, are considered nonexistent. For this reason, the model is called the "concerted," "all-or-none," or "symmetry-conserved" model. By fitting the oxygen saturation curve (Figure 10.21), we find that $L_0 = 9{,}000$ and $c = 0.014$. Thus, in the absence of oxygen, equilibrium greatly favors the T state by a factor of 9,000. On the other hand, the value of c shows that the binding of oxygen to a site in the R state is 1/0.014, or 71 times stronger than to one in the T state. As the above equations show, the progressive binding of oxygen changes the [T]/[R] ratio from 9,000 (no O_2 bound) to 126 (one O_2 bound), 1.76 (two O_2 bound), 0.25 (3 O_2 bound), and 0.00035 (4 O_2 bound).

The concerted model cannot account for negative homotropic cooperativity. (Negative cooperativity means that as a result of the first ligand binding, the second ligand would bind *less* readily. Glyceraldehyde-3-phosphate dehydrogenase, an important enzyme in glycolysis, exhibits this behavior.) Nevertheless, it is remarkable that the allosteric behavior of hemoglobin (and enzymes) can be described by just three equilibrium constants (L_0, K_R, and K_T).

The Sequential Model

An alternative model of cooperativity suggested by Koshland, Némethy, and Filmer* assumes that the affinity of vacant sites for a particular ligand changes progressively as sites are taken up. Referring to the binding of oxygen to hemoglobin, this means that when an oxygen molecule binds to a vacant site on one of the four subunits, the interaction causes the site to change its conformation, which in turn affects the binding constants of the three sites that are still vacant (Figure 10.22). For this reason, this model is called the *sequential model*. Unlike the concerted model, the sequential model can have tetrameters that consist of both R- and T-state subunits such as R_2T_2 or R_3T. This approach, too, predicts a sigmoidal curve. The affinity for O_2 molecules increases from left to right in Figure 10.22.

At present, the concerted and sequential models are both employed by biochemists in the study of enzymes. For hemoglobin, the actual mechanism seems more complex, and both models probably should be treated as limiting cases. In some cases, the sequential model has an advantage over the concerted model in that it can also account for negative homotropic cooperativity. Overall, these two models have

* D. E. Koshland, Jr., G. Némethy, and D. Filmer, *Biochemistry* **5**, 365 (1966).

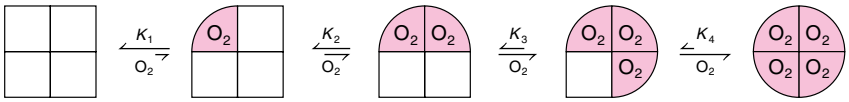


Figure 10.22
The sequential model for oxygen binding with hemoglobin. The squares represent the tense state; the quarter circles represent the relaxed state. The binding of a ligand to a subunit changes the conformation of that subunit (from T to R). This transition increases the affinity of the remaining subunits for the ligand. The dissociation constants decrease from K_1 to K_4.

provided biochemists with deeper insight into the structure and function of many enzymes.

Conformational Changes in Hemoglobin Induced by Oxygen Binding

Finally, we ask the question: If the four heme groups are well separated from one another in the hemoglobin molecule (the closest distance between any two Fe atoms is approximately 25 Å), then how are they able to transmit information regarding binding of oxygen? We can reasonably assume that the communication among the heme groups, called "heme–heme interaction," takes place by means of some kind of conformational change in the molecule. Deoxyhemoglobin and oxyhemoglobin are known to form different crystals. X-ray crystallographic studies show that there are indeed structural differences between the completely oxygenated and completely deoxygenated hemoglobin molecules. At present, an intense research effort is underway to understand how the binding of O_2 to one heme group can trigger such extensive structural changes from one subunit to another. Nature has apparently devised a most ingenious mechanism for cooperativity in hemoglobin. The Fe^{2+} ion in deoxyhemoglobin is in the high-spin state ($3d^6$, with four unpaired electrons),* and it is too large to fit into the plane of the porphyrin ring of the heme group (Figure 10.23). Consequently, the iron atom lies about 0.4 Å above a slightly domed porphyrin. Upon binding to O_2, the Fe^{2+} ion becomes low spin and shrinks sufficiently

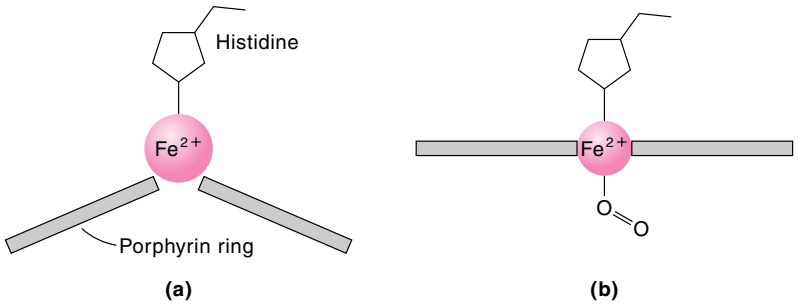


Figure 10.23
Schematic diagram showing the changes that occur when the heme group in hemoglobin binds an oxygen molecule. (a) The heme group in deoxyhemoglobin. The radius of the high-spin Fe^{2+} ion is too large to fit into the porphyrin ring. (b) When O_2 binds to Fe^{2+}, however, the ion shrinks somewhat so that it can fit in the plane of the ring. This movement pulls the histidine residue toward the ring and sets off a sequence of structural changes, thereby signaling the presence of an oxygen molecule at that heme group. The structural changes also drastically affect the affinity of the remaining heme groups for oxygen molecules.

* The electronic structure of the iron atom in the heme group is discussed in Chapter 12.

so that it can fit into the plane of the porphyrin ring, an arrangement that is energetically more favorable. (We can understand the change in size by recognizing that in the high-spin state, the 3*d* electrons are prohibited from coming too close to one another by the Pauli exclusion principle. Hence, the high-spin-state ion is larger than the low-spin-state ion.) When the Fe^{2+} ion moves into the porphyrin ring, it pulls the histidine ligand with it and sets off the chain of events that eventually lead to conformational changes in other parts of the molecule. This sequence is the means by which the binding of oxygen at one heme site is communicated to the other sites. To test this idea, chemists have replaced the histidine side chain with an imidazole ligand that resembles histidine but is detached from the polypeptide chain of the subunit:*

Imidazole

The movement of imidazole when oxygen is bound to the iron will have no effect on the conformation of the protein molecule. Indeed, results show that in this modified system, cooperativity is much attenuated but not totally eliminated. Apparently, other structural changes that are not yet fully understood also contribute to cooperativity.

10.7 pH Effects on Enzyme Kinetics

A useful way to understand the enzyme mechanism is to study the rate of an enzyme-catalyzed reaction as a function of pH. The activities of many enzymes vary with pH in a manner that can often be explained in terms of the dissociation of acids and bases. This is not too surprising because most active sites function as general acids and general bases in catalysis. Figure 10.24 shows a plot of the initial rate versus pH for the reaction catalyzed by the enzyme fumarase. As can be seen, the plot gives a bell-shaped curve. The pH at the maximum of the curve is called the pH *optimum*, which corresponds to the maximum activity of the enzyme; above or below this pH, the activity declines. Most enzymes that are active within cells have a pH optimum fairly close to the range of pH within which cells normally function. For example, the pH optima of two digestive enzymes, pepsin and trypsin, occur at about pH 2 and pH 8, respectively. The reasons are not hard to understand. Pepsin is secreted into the lumen of the stomach, where the pH is around 2. On the other hand, trypsin is secreted into and functions in the alkaline environment of the intestine, where the pH

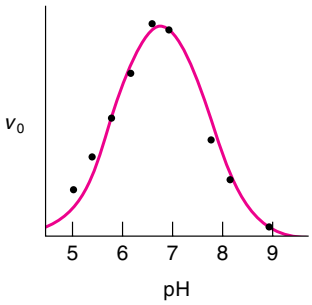
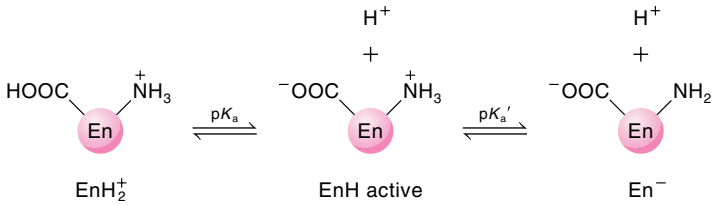


Figure 10.24
The effect of pH on the initial rate of the reaction catalyzed by the enzyme fumarase. [After C. Tanford, *Physical Chemistry of Macromolecules.* Copyright 1967 by John Wiley & Sons. Reprinted by permission of Charles Tanford.]

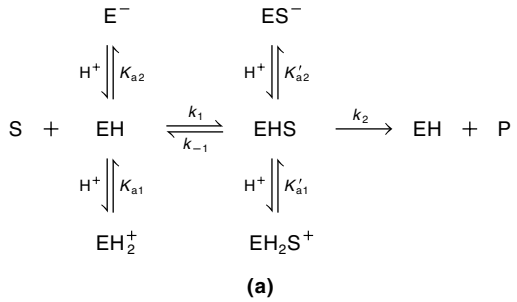
* See D. Barrick, N. T. Ho, V. Simplaceanu, F. W. Dahlquist, and C. Ho, *Nat. Struct. Biol.* **4**, 78 (1997).

is about 8. For general assays of enzyme activity, then, the solution should be buffered at the pH optimum for catalysis. Finally, when studying the influence of pH on enzyme activity, we should be careful to avoid gross structural changes brought about by the large changes in pH, such as protein denaturation.

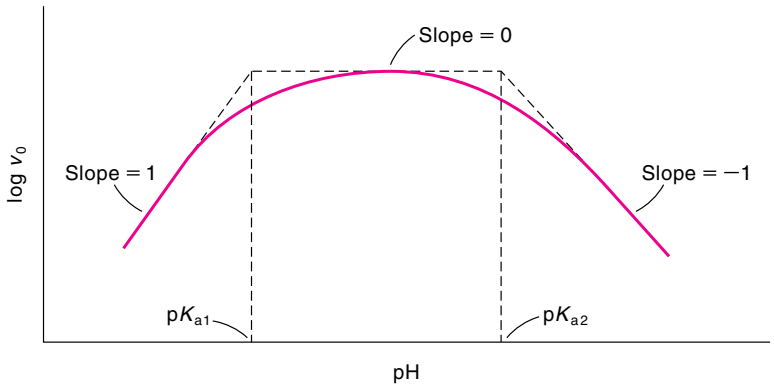
The initial rate versus pH plot shown in Figure 10.24 yields much useful kinetic and mechanistic information about enzyme catalysis. In the simplest case, let us assume that an enzyme has two dissociable protons (say, from the –COOH and $-NH_3^+$ groups) with the zwitterion as the active form:



The concentration of the EnH form goes through a maximum as the pH is varied, so that the rate also passes through a maximum. The enzyme–substrate complex also may exist in three states of dissociation (as in the case of the free enzyme), with only the intermediate form capable of giving rise to products. Figure 10.25a shows the kinetic scheme for this reaction. At low substrate concentrations, the enzyme exists



(a)



(b)

Figure 10.25
Effect of pH on enzyme kinetics. (a) Reaction scheme for the enzyme-catalyzed reaction. (b) Plot of $\log v_0$ versus pH according to Equations 10.34, 10.35, and 10.36. At the intercept of the lines with slopes 1 and 0, pH = pK_{a1}. Similarly, at the intercept of the lines with slopes −1 and 0, pH = pK_{a2}.

mostly in the free form; therefore, the pH is controlled by the dissociation of the free enzyme. Thus, analysis of the experimental pH dependence of the initial rate at low substrate concentrations provides information about pK_{a1} and pK_{a2} of the free enzyme. On the other hand, at high substrate concentrations, when the enzyme is saturated with substrate, analysis of pH dependence allows the determination of pK'_{a1} and pK'_{a2}, which relate to the dissociation of the enzyme–substrate complex.

In Figure 10.25a the rate is given by*

$$v_0 = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S}\left(1 + \dfrac{K_{a2}}{[\mathrm{H}^+]} + \dfrac{[\mathrm{H}^+]}{K_{a1}}\right) + [\mathrm{S}]\left(1 + \dfrac{K'_{a2}}{[\mathrm{H}^+]} + \dfrac{[\mathrm{H}^+]}{K'_{a1}}\right)} \tag{10.32}$$

At low (and constant) substrate concentrations, we can ignore the second term in the denominator in Equation 10.32, so that

$$v_0 = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S}\left(1 + \dfrac{K_{a2}}{[\mathrm{H}^+]} + \dfrac{[\mathrm{H}^+]}{K_{a1}}\right)} \tag{10.33}$$

Consider the following three cases:

CASE 1. At low pH or high $[\mathrm{H}^+]$, the term $[\mathrm{H}^+]/K_{a1}$ predominates in the denominator of Equation 10.33, and we write

$$v_0 = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]K_{a1}}{K_\mathrm{S}[\mathrm{H}^+]}$$

or

$$\begin{aligned} \log v_0 &= \log\frac{k_2[\mathrm{E}]_0[\mathrm{S}]K_{a1}}{K_\mathrm{S}} - \log[\mathrm{H}^+] \\ &= \text{constant} + \mathrm{pH} \end{aligned} \tag{10.34}$$

Thus, a plot of $\log v_0$ versus pH yields a straight line with a slope of $+1$ at low pH values.

CASE 2. At high pH or low $[\mathrm{H}^+]$, the $K_{a2}/[\mathrm{H}^+]$ term predominates in the denominator of Equation 10.33, and we have

$$v_0 = \frac{k_2[\mathrm{E}]_0[\mathrm{S}][\mathrm{H}^+]}{K_\mathrm{S}K_{a2}}$$

or

$$\begin{aligned} \log v_0 &= \log\frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S}K_{a2}} + \log[\mathrm{H}^+] \\ &= \text{constant} - \mathrm{pH} \end{aligned} \tag{10.35}$$

In this case, a plot of $\log v_0$ versus pH gives a straight line with a slope of -1.

* For the derivation, see Reference 3 on p. 6.

Table 10.3
pK_a Values of Amino Acids

Side Chain	Free State	Active Site	Enzyme
Glu	3.9	6.5	Lysozyme
His	6.0	5.2	Ribonuclease
Cys	8.3	4.0	Papain
Lys	10.8	5.9	Acetoacetate decarboxylase

CASE 3. At intermediate pH values, the first term (that is, 1) is the predominant term in the denominator of Equation 10.33. Therefore,

$$v_0 = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S}}$$

or

$$\log v_0 = \log \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{S}} \tag{10.36}$$

Because the term on the right is a constant, $\log v_0$ is *independent* of pH. The plot in Figure 10.25b shows these three situations and the determination of pK_{a1} and pK_{a2}.

Two points are worth noting. First, the above treatment is based on Michaelis–Menten kinetics. In reality, there may be more intermediates with additional dissociation constants, even for a one-substrate reaction. Second, as Table 10.3 shows, the pK_a values for the amino acid residues at the active site can be quite different from those of the corresponding free amino acids in solution (see Table 8.6). This deviation in pK_a values is the result of hydrogen bonding, electrostatic, and other types of interactions at the active site. Thus, as a rule, we do not rely solely on pK_a values to identify amino acids in enzyme catalysis; often pH dependence measurements are used in conjunction with spectroscopic and X-ray diffraction studies to construct a three-dimensional picture of the active site.

Suggestions for Further Reading

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Problems

Michaelis–Menten Kinetics

10.1 Explain why a catalyst must affect the rate of a reaction in both directions.

10.2 Measurements of a certain enzyme-catalyzed reaction give $k_1 = 8 \times 10^6\ M^{-1}\ s^{-1}$, $k_{-1} = 7 \times 10^4\ s^{-1}$, and $k_2 = 3 \times 10^3\ s^{-1}$. Does the enzyme–substrate binding follow the equilibrium or steady-state scheme?

10.3 The hydrolysis of acetylcholine is catalyzed by the enzyme acetylcholinesterase, which has a turnover rate of 25,000 s^{-1}. Calculate how long it takes for the enzyme to cleave one acetylcholine molecule.

10.4 Derive the following equation from Equation 10.10,

$$\frac{v_0}{[S]} = \frac{V_{max}}{K_M} - \frac{v_0}{K_M}$$

and show how you would obtain values of K_M and V_{max} graphically from this equation.

10.5 An enzyme that has a K_M value of $3.9 \times 10^{-5}\ M$ is studied at an initial substrate concentration of 0.035 M. After 1 min, it is found that 6.2 μM of product has been produced. Calculate the value of V_{max} and the amount of product formed after 4.5 min.

10.6 The hydrolysis of *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) to *p*-nitroaniline and *N*-glutaryl-L-phenylalanine is catalyzed by α-chymotrypsin. The following data are obtained:

$[S]/10^{-4}\ M$	2.5	5.0	10.0	15.0
$v_0/10^{-6}\ M \cdot min^{-1}$	2.2	3.8	5.9	7.1

where [S] = GPNA. Assuming Michaelis–Menten kinetics, calculate the values of V_{max}, K_M, and k_2 using the Lineweaver–Burk plot. Another way to treat the data is to plot v_0 versus $v_0/[S]$, which is the Eadie–Hofstee plot. Calculate the values of V_{max}, K_M, and k_2 from the Eadie–Hofstee treatment, given that $[E]_0 = 4.0 \times 10^{-6}\ M$. [*Source*: J. A. Hurlbut, T. N. Ball, H. C. Pound, and J. L. Graves, *J. Chem. Educ.* **50**, 149 (1973).]

10.7 The K_M value of lysozyme is $6.0 \times 10^{-6}\ M$ with hexa-*N*-acetylglucosamine as a substrate. It is assayed at the following substrate concentrations: **(a)** $1.5 \times 10^{-7}\ M$, **(b)** $6.8 \times 10^{-5}\ M$, **(c)** $2.4 \times 10^{-4}\ M$, **(d)** $1.9 \times 10^{-3}\ M$, and **(e)** 0.061 M. The initial rate measured at 0.061 M was 3.2 $\mu M\ min^{-1}$. Calculate the initial rates at the other substrate concentrations.

10.8 The hydrolysis of urea,

$$(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$$

has been studied by many researchers. At 100°C, the (pseudo) first-order rate constant is $4.2 \times 10^{-5}\ s^{-1}$. The reaction is catalyzed by the enzyme urease, which at 21°C has a rate constant of $3 \times 10^4\ s^{-1}$. If the enthalpies of activation for the uncatalyzed and catalyzed reactions are 134 kJ mol^{-1} and 43.9 kJ mol^{-1}, respectively, **(a)** calculate the temperature at which the nonenzymatic hydrolysis of urea would proceed at the same rate as the enzymatic hydrolysis at 21°C; **(b)** calculate the lowering of $\Delta G^{\ddagger}$ due to urease; and **(c)** comment on the sign of $\Delta S^{\ddagger}$. Assume that $\Delta H^{\ddagger} = E_a$ and that $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are independent of temperature.

10.9 An enzyme is inactivated by the addition of a substance to a solution containing the enzyme. Suggest three ways to find out whether the substance is a reversible or an irreversible inhibitor.

10.10 Silver ions are known to react with the sulfhydryl groups of proteins and therefore can inhibit the action of certain enzymes. In one reaction, 0.0075 g of $AgNO_3$ is needed to completely inactivate a 5-mL enzyme solution. Estimate the molar mass of the enzyme. Explain why the molar mass obtained represents the minimum value. The concentration of the enzyme solution is such that 1 mL of the solution contains 75 mg of the enzyme.

10.11 The initial rates at various substrate concentrations for an enzyme-catalyzed reaction are as follows:

$[S]/M$	$v_0/10^{-6}\ M \cdot min^{-1}$
2.5×10^{-5}	38.0
4.00×10^{-5}	53.4
6.00×10^{-5}	68.6
8.00×10^{-5}	80.0
16.0×10^{-5}	106.8
20.0×10^{-5}	114.0

(a) Does this reaction follow Michaelis–Menten kinetics? **(b)** Calculate the value of V_{max} of the reaction. **(c)** Calculate the K_M value of the reaction. **(d)** Calculate the initial rates at $[S] = 5.00 \times 10^{-5}\ M$ and $[S] = 3.00 \times 10^{-1}\ M$. **(e)** What is the total amount of product formed during the first 3 min at $[S] = 7.2 \times 10^{-5}\ M$? **(f)** How would an increase in the enzyme concentration by a factor of 2 affect each of the following quantities: K_M, V_{max}, and v_0 (at $[S] = 5.00 \times 10^{-5}\ M$)?

10.12 An enzyme has a K_M value of $2.8 \times 10^{-5}\ M$ and a V_{max} value of 53 $\mu M\ min^{-1}$. Calculate the value of v_0 if $[S] = 3.7 \times 10^{-4}\ M$ and $[I] = 4.8 \times 10^{-4}\ M$ for **(a)** a competitive inhibitor, **(b)** a noncompetitive inhibitor, and **(c)** an uncompetitive inhibitor. ($K_I = 1.7 \times 10^{-5}\ M$ for all three cases.)

10.13 The degree of inhibition i is given by $i\% = (1 - \alpha)100\%$, where $\alpha = (v_0)_{\text{inhibition}}/v_0$. Calculate the percent inhibition for each of the three cases in Problem 10.12.

10.14 An enzyme-catalyzed reaction ($K_M = 2.7 \times 10^{-3}\ M$) is inhibited by a competitive inhibitor I ($K_I = 3.1 \times 10^{-5}\ M$). Suppose that the substrate concentration is $3.6 \times 10^{-4}\ M$. How much of the inhibitor is needed for 65% inhibition? How much does the substrate concentration have to be increased to reduce the inhibition to 25%?

10.15 Calculate the concentration of a noncompetitive inhibitor ($K_I = 2.9 \times 10^{-4}\ M$) needed to yield 90% inhibition of an enzyme-catalyzed reaction.

10.16 Derive Equation 10.22.

10.17 The metabolism of ethanol in our bodies is catalyzed by liver alcohol dehydrogenase (LADH) to acetaldehyde and finally to acetate. In contrast, methanol is converted to formaldehyde (also catalyzed by LADH), which can cause blindness or even death. An antidote for methanol is ethanol, which acts as a competitive inhibitor for LADH. The excess methanol can then be safely discharged from the body. How much absolute (100%) ethanol would a person have to consume after ingesting 50 mL of methanol (a lethal dosage) to reduce the activity of LADH to 3% of the original value? Assume that the total fluid volume in the person's body is 38 liters and that the densities of ethanol and methanol are 0.789 g mL^{-1} and 0.791 g mL^{-1}, respectively. The K_M value for methanol is $1.0 \times 10^{-2}\ M$, and the K_I value for ethanol is $1.0 \times 10^{-3}\ M$. State any assumptions.

Allosteric Interactions

10.18 **(a)** What is the physiological significance of cooperative O_2 binding by hemoglobin? Why is O_2 binding by myoglobin not cooperative? **(b)** Compare the concerted model with the sequential model for the binding of oxygen with hemoglobin.

10.19 Fatality usually results when more than 50% of a human being's hemoglobin is complexed with carbon monoxide. Yet a person whose hemoglobin content is diminished by anemia to half its original content can often function normally. Explain.

10.20 Competitive inhibitors, when present in small amounts, often act as activators to allosteric enzymes. Why?

10.21 What is the advantage of having the heme group in a hydrophobic region in the myoglobin and hemoglobin molecule?

10.22 What is the effect of each of the following actions on oxygen affinity of adult hemoglobin (Hb A) *in vitro*? **(a)** Increase pH, **(b)** increase partial pressure of CO_2, **(c)** decrease [BPG], **(d)** dissociate the tetramer into monomers, and **(e)** oxidize Fe(II) to Fe(III).

10.23 Although it is possible to carry out X-ray diffraction studies of fully deoxygenated hemoglobin and fully oxygenated hemoglobin, it is much more difficult, if not impossible, to obtain crystals in which each hemoglobin molecule is bound to only one, two, or three oxygen molecules. Explain.

10.24 When deoxyhemoglobin crystals are exposed to oxygen, they shatter. On the other hand, deoxymyoglobin crystals are unaffected by oxygen. Explain.

Additional Problems

10.25 An enzyme contains a single dissociable group at its active site. For catalysis to occur, this group must be in the dissociated (that is, negative) form. The substrate bears a net positive charge. The reaction scheme can be represented by

$$EH \rightleftharpoons H^+ + E^-$$
$$E^- + S^+ \rightleftharpoons ES \rightarrow E + P$$

(a) What kind of inhibitor is H^+? **(b)** Write an expression for the initial rate of the reaction in the presence of the inhibitor.

10.26 The discovery in the 1980s that certain RNA molecules (the ribozymes) can act as enzymes was a surprise to many chemists. Why?

10.27 The activation energy for the decomposition of hydrogen peroxide,

$$2H_2O_2(aq) \rightarrow 2H_2O(l) + O_2(g)$$

is 42 kJ mol^{-1}, whereas when the reaction is catalyzed by the enzyme catalase, it is 7.0 kJ mol^{-1}. Calculate the temperature that would cause the nonenzymatic catalysis to proceed as rapidly as the enzyme-catalyzed decomposition at 20°C. Assume the pre-exponential factor to be the same in both cases.

10.28 Referring to the concerted model discussed on p. 390, show that $K_1 = cL_0$.

10.29 The following data were obtained for the variation of V_{max} with pH for a reaction catalyzed by α-amylase at 24°C. What can you conclude about the pK_a values of the ionizing groups at the active site?

pH	3.0	3.5	4.0	4.5	5.0	5.5	
V_{max} (arbitrary units)	200	501	1584	1778	3300	5248	
pH	6.0	6.5	7.0	7.5	8.0	8.5	9.0
V_{max} (arbitrary units)	5250	5251	2818	2510	1585	398	158

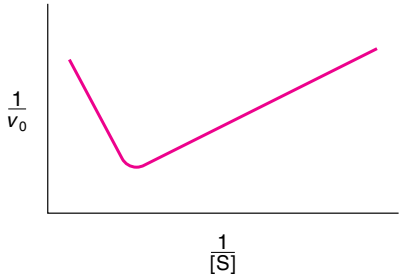
10.30 **(a)** Comment on the following data obtained for an enzyme-catalyzed reaction (no calculations are needed):

t/°C	10	15	20	25	30	35	40	45
V_{max} (arbitrary units)	1.0	1.7	2.3	2.6	3.2	4.0	2.6	0.2

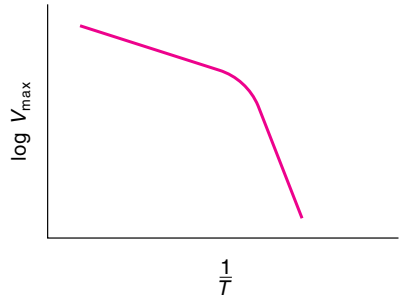
(b) Referring to Equation 10.8, under what conditions will an Arrhenius plot (that is, $\ln k$ versus $1/T$) yield a straight line?

10.31 Crocodiles can be submerged in water for a prolonged period of time (up to an hour), while drowning their preys. It is known that BPG does not bind to the crocodile deoxyhemoglobin but the bicarbonate ion does. Explain how this action enables crocodiles to utilize practically all of the oxygen bound to hemoglobin.

10.32 Give an explanation for the Lineweaver–Burk plot for a certain enzyme-catalyzed reaction shown below.



10.33 The following Arrhenius plot has been obtained for a certain enzyme. Account for the shape of the plot.



10.34 In Lewis Carroll's tale "Through the Looking Glass," Alice wonders whether looking-glass milk on the other side of the mirror would be fit to drink. What do you think?

10.35 Referring to Problem 9.64, calculate k_D for an enzyme–substrate reaction such as that between an enzyme ($D \approx 4 \times 10^{-7}$ cm^2 s^{-1}) and a substrate ($D \approx 5 \times 10^{-6}$ cm^2 s^{-1}) at 20°C. The distance between the enzyme and the substrate may be taken as 5×10^{-8} cm. Compare your result with the k_{cat}/K_M values listed in Table 10.1.

10.36 When fruits such as apples and pears are cut, the exposed areas begin to turn brown. This is the result of an oxidation reaction. Often the browning action can be prevented or slowed by adding a few drops of lemon juice. What is the chemical basis for this treatment?

10.37 "Dark meat" (the legs) and "white meat" (the breast) are one's choices when eating a turkey. Explain what causes the meat to assume different colors.

10.38 Despite what you may have read in science fiction novels or seen in horror movies, it is extremely unlikely that insects can ever grow to human size. Why?

10.39 The first-order rate constant for the dehydration of carbonic acid,

$$H_2CO_3(aq) \rightleftharpoons CO_2(g) + H_2O(l)$$

is about 1×10^2 s^{-1}. In view of this rather high rate constant, explain why it is necessary to have the enzyme carbonic anhydrase to enhance the rate of dehydration in the lungs.

10.40 Referring to Equation 10.12, sketch the Eadie–Hofstee plots for **(a)** a competitive inhibitor and **(b)** a noncompetitive inhibitor.