Henri–Michaelis–Menten Equation

15

A rate equation (or the rate law) gives the experimentally observed dependence of rate on the concentration of reactants. Rate equations are at the heart of any kinetic study as they help us describe the system in a mathematical formalism. This is true for enzyme catalysis as well. Besides its aesthetic beauty, the compact mathematical description of reaction kinetics serves the twin purposes of qualitative description of the system and quantitative evaluation of rate constants. An early attempt to capture the kinetics of enzyme catalysis was made by Victor Henri (in 1903, Chap. 2), and this was subsequently developed by Leonor Michaelis and Maud Menten (in 1913). The rate equation so described is a fundamental equation of enzyme kinetics and goes by the name Henri–Michaelis–Menten equation. It is more commonly referred to as Michaelis–Menten equation. The derivation of the rate equation for a simple, single-substrate enzymatic reaction is especially instructive. In the process, it describes the general logic used to derive such rate equations – an exercise central to any enzyme kinetic study. This chapter will describe the development, significance, and salient features of the Michaelis–Menten equation.

15.1 Derivation of the Michaelis–Menten Equation

Initial clues to understand enzyme-catalyzed reaction rates came from the saturation effect. At a constant $[E_t]$, the reaction rate increases with increasing $[S]$ until it reaches a limiting, maximum value (Fig. 11.2). In contrast, reaction rate increases linearly with increasing $[S]$ in uncatalyzed reactions. The *saturation effect* observed with enzyme-catalyzed reactions led to the postulation of an enzyme–substrate (ES) complex – formed when enzyme and substrate come together through diffusion. The product is formed and released from ES complex (this means, $ES \rightarrow E + P$) and not directly from the substrate. We can then represent the simplest general scheme for one substrate-one product reaction as shown (Fig. 15.1).

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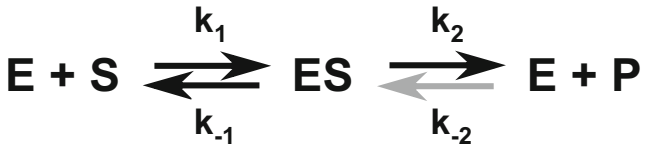

Fig. 15.1 Simplest enzyme reaction scheme for $S \rightarrow P$ conversion invoking the formation of a single *ES* complex. The forward rate constants for the first and the second step are shown as k_1 and k_2, respectively. Reverse rate constants for the corresponding steps are shown as k_{-1} and k_{-2}

According to this representation, the enzyme-catalyzed rate will be directly related to [*ES*] and indirectly related to [*S*]. Taking this into account, we may write the rate of enzyme-catalyzed reaction (velocity "*v*") in terms of *ES* concentration as shown:

$$v = \frac{d[P]}{d\mathrm{t}} = k_2 \times [ES]$$

Deriving the Rate Equation Thus Becomes an Exercise in Evaluating [ES]. In order to obtain a useful rate equation, it is necessary to obtain [*ES*] in terms of [*S*] at any given instance. But this is not a trivial matter, because, as we have noted earlier, concentrations of *S*, *P*, *E*, and *ES* are all changing as a function of reaction time (Fig. 11.1, Chap. 11). A conceptual breakthrough in simplifying this difficulty was made by setting up well-defined initial conditions and making certain clear assumptions. Three important experimental conditions are as follows:

1. All experimental conditions like pH, temperature, ionic strength, etc. remain constant throughout the course of experiment.
2. Enzyme being a catalyst, its concentration is very much lower than the concentration of substrate. Typically, concentration of the substrate is at least 1000 times higher than that of the enzyme. This permits us to approximate $[S_t] \approx [S]$, although $[S_t] = [S] + [ES]$.
3. Strictly the initial rate (velocity "*v*") is recorded. This is the rate at the beginning of the reaction or the instantaneous rate extrapolated to time zero. This will be the unbiased rate when $[P] \approx 0$.

Obviously, additional assumptions are clearly needed to evaluate [*ES*] and then to obtain the rate equation.

The Equilibrium Assumption Michaelis and Menten (and of course Victor Henri before them!) provided the conceptual breakthrough and derived the now famous rate equation for an enzyme-catalyzed reaction. They assumed that the formation of

ES complex from *E* and *S* is at equilibrium; accordingly k_2 is considered to be much smaller in magnitude when compared to k_1 and k_{-1} (Fig. 15.1). With this equilibrium assumption and the experimental conditions listed above, it was possible to evaluate [*ES*] as below:

$$K_{\text{eq}} = \frac{[E][S]}{[\text{ES}]} = \frac{k_{-1}}{k_1} \quad \textit{and hence } k_1[E][S] = k_{-1}[\text{ES}]$$

However, $[E_t] = [E] + [ES]$ and therefore substituting for [*E*] we get

$$k_1[E_t][S] - k_1[ES][S] = k_{-1}[ES]$$

On simplification,

$$[ES] = \frac{k_1[E_t][S]}{(k_{-1} + k_1[S])} = \frac{[E_t][S]}{\left(\frac{k_{-1}}{k_1} + [S]\right)}$$

Substituting this value of [*ES*] in the rate equation, $v = k_2 \times [ES]$, we get.

$$v = \frac{k_2[E_t][S]}{\left(\frac{k_{-1}}{k_1} + [S]\right)}$$

By defining $V_{\max}$ (as $k_2 \times [E_t]$) and K_S (as k_{-1}/k_1), this takes the original form of Michaelis–Menten equation:

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

It is obvious that when all the enzyme is in *ES* form (i.e., $[ES] = [E_t]$), the reaction velocity reaches a limiting maximum value (i.e., $v = V_{\max}$). Also, the constant K_S (the Michaelis constant) is nothing but the equilibrium (dissociation) constant – in accordance with the equilibrium assumption. Rate constant k_{-2} (see Fig. 15.1) does not appear in the final form of the rate equation. The rate "$k_{-2} \times [P]$" equals zero as long as $[P] \approx 0$ (initial velocity conditions are met!).

The Steady-State Assumption In the equilibrium assumption (described above), the binding step ($E + S \rightleftarrows ES$) was set at equilibrium, by explicitly assuming that k_2 is quite small. It may not be always true that k_2 is negligible (when compared to k_1 and k_{-1} in Fig. 15.1). In such situations, "equilibrium assumption" is invalid and cannot be used to evaluate [*ES*]. Briggs and Haldane (1925) overcame this limitation by suggesting a more general approach. They viewed the [*ES*] to be at steady state (see Chap. 10 and Fig. 11.1). As the $[S_t]/[E_t]$ ratio increases, the steady-state region occupies increasing fraction of the total reaction time. Accordingly, concentration of

ES remains unchanged – because its formation and disappearance rates are equal (i.e., $d[ES]/dt \approx 0$). With this assumption and from Fig. 15.1, we can set up a balanced equation and solve for [*ES*].

$$\text{Rate of } ES \text{ formation} = \text{rate of } ES \text{ disappearance}$$

and therefore,

$$k_1[E][S] = k_{-1}[ES] + k_2[ES]$$

However, $[E_t] = [E] + [ES]$ and therefore substituting for [*E*], we get

$$k_1[E_t][S] - k_1[ES][S] = k_{-1}[ES] + k_2[ES]$$

On simplification,

$$[ES] = \frac{k_1[E_t][S]}{k_{-1} + k_2 + k_1[S]} = \frac{[E_t][S]}{\left(\frac{k_{-1}+k_2}{k_1}\right) + [S]}$$

Since velocity (v) is $= k_2 \times [ES]$ and substituting for [*ES*],

$$v = \frac{k_2[E_t][S]}{\left(\frac{k_{-1}+k_2}{k_1}\right) + [S]}$$

$$v = \frac{V_{\max}[S]}{K_M + [S]} \qquad \textbf{Michaelis} - \textbf{Mentenequation}$$

As before, we define V_{max} (as $k_2 \times [E_t]$); however $K_M = (k_{-1} + k_2)/k_1$ is a lumped up constant (commonly referred to as "Michaelis constant") arising from the three rate constants.

Equilibrium Assumption Is a Limiting Case of Steady-State Assumption Regardless of whether one uses equilibrium assumption or steady-state assumption (or considers $d[ES]/dt$ as very small!), we arrive at an equation that is isomorphic with the original Michaelis–Menten equation – which is an equation describing a rectangular hyperbola. The two representations of the enzyme rate equation differ in the nature/composition of the Michaelis constant. According to steady-state assumption, we see that

$$\begin{aligned} K_M &= (k_{-1} + k_2)/k_1 \\ &= (k_{-1}/k_1) + (k_2/k_1) \\ &= K_S + (k_2/k_1) \end{aligned}$$

For an enzyme $K_M = K_S$, whenever the k_2 is very small compared to k_1 (i.e., $k_2/k_1 \approx 0$). Clearly, the equilibrium assumption is a limiting case of steady-state assumption.

15.2 Salient Features of Michaelis-Menten Equation

The derivation of Michaelis–Menten equation describes the general logic used to derive enzymatic rate equations. It establishes the rate law for an isolated enzyme-catalyzed reaction under clearly specified conditions. Despite the assumptions involved, this elegant equation has stood the test of time. We will analyze the salient features and limits of this equation.

Anatomy of the Equation and its Limits The $v \rightarrow [S]$ plot form of Michaelis–Menten equation is a rectangular hyperbola. This adequately describes the characteristic saturation effect (Fig. 11.2) observed with enzyme catalysis. Enzyme behavior at the two limiting cases of [S] may also be visualized.

(i) When $[S]$ is very small compared to K_M (i.e., $K_M + [S] \approx K_M$, in the denominator), the equation takes the form

$$v = (V_{max}/K_M) \times [S]^{\mathbf{1}}$$

For very small values of $[S]$, therefore, the reaction is of first order with respect to "S." By analogy, V_{max}/K_M represents the first-order rate constant for this reaction (Table 15.1).

(ii) When $[S]$ is very large compared to K_M (i.e., $K_M + [S] \approx [S]$, in the denominator), the equation takes the form

$$v = (V_{max}) \times [S]^{\mathbf{0}}$$

For very large values of $[S]$, therefore, the reaction is of zero order with respect to "S." By analogy, V_{max} represents the zero-order rate constant for this reaction.

Table 15.1 Order of an enzyme-catalyzed reaction changes with increasing [*S*]

[*S*]	Reaction order	Rate constant	Units
$[S] << K_M$	First	V_{max}/K_M	$M^0 \times sec^{-1}$
[*S*] around K_M	Fractional	–	–
$[S] >> K_M$	Zero	V_{max}	$M^1 \times sec^{-1}$

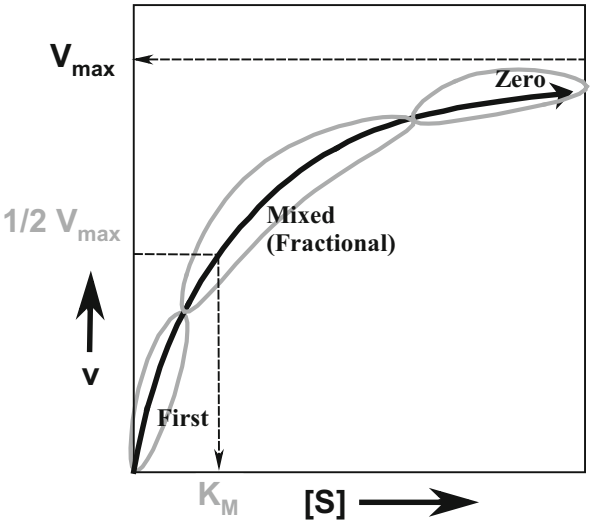

Fig. 15.2 Order of an enzyme-catalyzed reaction gradually changes from first order to zero order as [*S*] increases from zero to infinity. A schematic representation is shown

Obviously, the reaction order for an enzyme-catalyzed reaction changes when we move from sub-saturating to saturating levels of [*S*]. As we go from very low to very high [*S*], it follows the sequence, first order → mixed (fractional) order → zero order, as shown schematically in Fig. 15.2.

Enzyme Kinetic Constants and Their Units Both V_{max} and K_M (and therefore V_{max}/K_M) are constants for a given enzyme. The units for K_M and V_{max} will be those in which [*S*] and *v*, respectively, are measured.

Since velocity "*v*" can reach a maximum of V_{max} (as $[S] \rightarrow \infty$, see Fig. 15.2), they have similar units. Just like enzyme reaction velocity, V_{max} is expressed as μmol product formed$\times min^{-1} \times mg^{-1}$ protein or $U \times mg^{-1}$ protein. By definition, $V_{max} = k_2 \times [E_t]$, and therefore V_{max} is a constant provided the amount of enzyme catalyst ($[E_t]$) is fixed. We notice that the constant k_2 *is an intensive property* and is intrinsic for a given enzyme. However, $[E_t]$ *is an extensive property* whose magnitude can be adjusted. The amount of enzyme protein can also be expressed as number of moles of that enzyme (Chap. 14). In this form, the constant k_2 (which equals $V_{max}/[E_t]$) is more generally denoted as k_{cat} – the turnover number. It has the units of time^{-1} as expected of a zero-order rate constant (Table 15.1).

The units for K_M and [*S*] are the same (i.e., concentration such as mM, μM, etc.). This is expected when we consider K_M purely as a dissociation constant (recall the

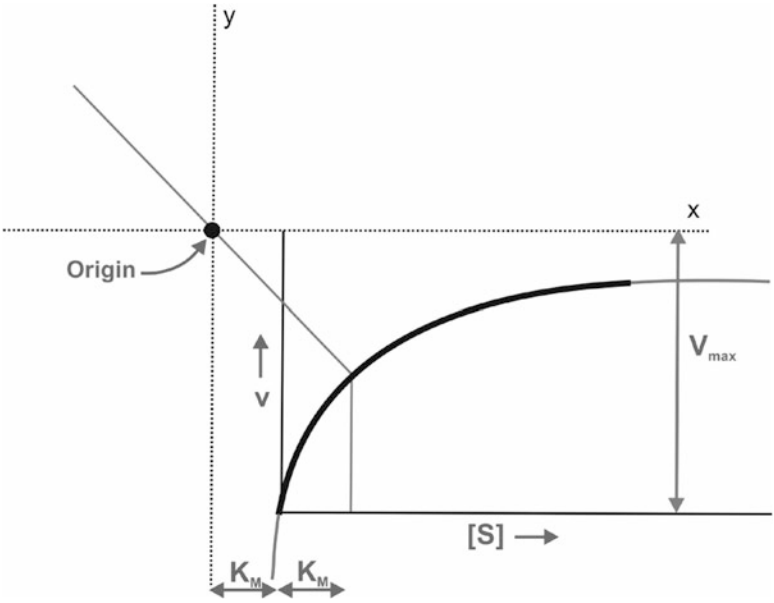

Fig. 15.3 The $v \rightarrow [S]$ curve described by the Michaelis–Menten equation is part of a rectangular hyperbola. The highlighted short arc (dark line) is the actual region where experimental observations for an enzyme are physically possible. The two limbs of this rectangular hyperbola form asymptotes of an infinite curve as shown. This makes direct estimation of both K_M and V_{max} values difficult

K_S of equilibrium assumption). Numerically, K_M is equal to $[S]$ at which the reaction rate is half its maximal value. This is obvious when we substitute velocity by $V_{max}/2$ and simplify the Michaelis–Menten equation. By corollary, both K_M and $[S]$ are expressed in concentration units.

Relation to Rectangular Hyperbolic Geometry The Michaelis–Menten equation describes an equation for rectangular hyperbola. Enzyme reactions that follow such a rate law display $v \rightarrow [S]$ curves tracing a rectangular hyperbola (Fig. 15.3).

It is a geometric property of rectangular hyperbola (from mathematics) that, despite the absolute magnitudes of X- and Y-axis scales, the ratio of X-axis coordinates corresponding to any two Y-axis coordinates remains constant. Therefore, regardless of absolute values of the kinetic constants (viz., V_{max} and K_M), the ratio of $[S]$ taken at any two fixed fractional saturations (v/V_{max}) remains same. For example, the ratio of $[S]$ at 0.9 saturation (i.e., $[S]$ when $v = 0.9\ V_{max}$) to $[S]$ at 0.1 saturation (i.e., $[S]$ when $v = 0.1\ V_{max}$) is 81 – a constant (as shown in the box below). We can demonstrate this for many other substrate saturation ratios such as $[S]_{0.8}/[S]_{0.2} = 16$ and $[S]_{0.9}/[S]_{0.2} = 36$, etc.

Calculating $[S]_{0.9}/[S]_{0.1}$

The Michaelis–Menten equation may be rearranged for $[S]$ as shown:

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

Substituting v by 0.9 V_{max} and solving for $[S]_{0.9}$,

$$[S]_{0.9} = \frac{0.9\,V_{max}}{V_{max} - 0.9\,V_{max}} K_M = \frac{0.9}{0.1} K_M = 9\,K_M$$

Similarly, substituting v by 0.1 V_{max} and solving for $[S]_{0.1}$,

$$[S]_{0.1} = \frac{0.1\,V_{max}}{V_{max} - 0.1\,V_{max}} K_M = \frac{0.1}{0.9} K_M = \frac{1}{9} K_M$$

Taking ratios,

$$\mathbf{[S]_{0.9}/[S]_{0.1} = 81}$$

If $[S]_{0.9}/[S]_{0.1} = 81$, then that enzyme kinetic data fits the Michaelis–Menten equation. What if $[S]_{0.9}/[S]_{0.1}$ is not 81? Obviously such an enzyme does not obey the Michaelis–Menten kinetics, and the data points do not trace a rectangular hyperbola (Choudhury and Punekar 2009). There are several possible causes of such behavior. Common examples of non-hyperbolic substrate saturation include inhibition by high $[S]$ and cooperative kinetics (Fig. 15.4). Such departures from simple hyperbolic behavior (as predicted by Michaelis–Menten equation) are also very informative. They are helpful in (a) deducing the kinetic mechanism involved (Chap. 23; Substrate Inhibition) or (b) establishing the phenomenon of cooperativity and allosteric regulation (Chap. 37; Regulation of Enzyme Activity).

Cooperative Kinetics E and S interactions are considered cooperative when the binding of one molecule of substrate to the enzyme can either facilitate (*positive cooperativity*) or hinder (*negative cooperativity*) the binding of subsequent molecules of the same substrate. Binding of oxygen to hemoglobin (an honorary enzyme!) is an early example of positive cooperativity (Hill 1910). A sigmoid saturation curve (unlike the rectangular hyperbola traced by Michaelis–Menten equation) is better described by the Hill equation:

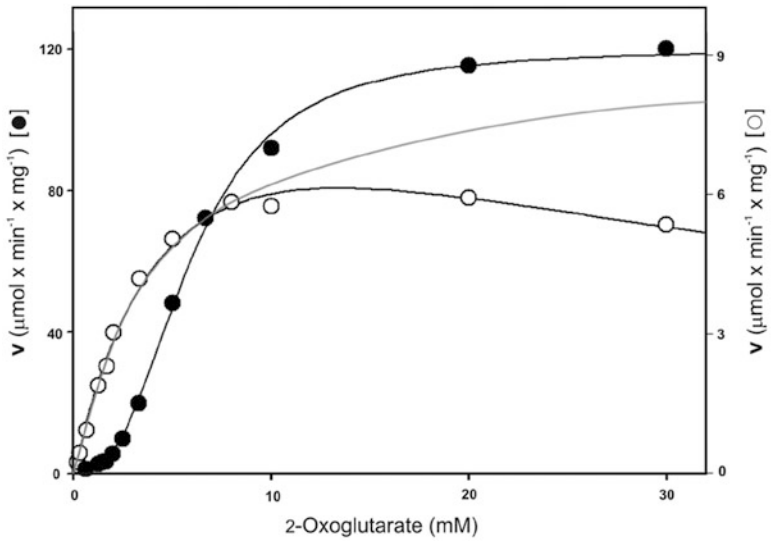

Fig. 15.4 Two examples of non-hyperbolic substrate saturation. NADP-glutamate dehydrogenase from *Aspergillus niger* [●] shows sigmoid 2-oxoglutarate (substrate) saturation. The same enzyme from *Aspergillus terreus* [○] however exhibits substrate inhibition. A theoretical curve (in gray) describing the Michaelis–Menten kinetics is included for comparison

$$v = \frac{V_{\max}[S]^h}{{K_{0.5}}^h + [S]^h} \qquad \textbf{Hill equation}$$

where $[S]$ is the substrate concentration, v is the corresponding initial velocity, and $\boldsymbol{h}$ (also denoted as $\boldsymbol{n_H}$) is the *Hill coefficient*. The constant $\boldsymbol{K_{0.5}}$ is analogous to K_M of the Michaelis–Menten equation. It defines the value of $[S]$ at which $v = V_{max}/2$, but it is not a Michaelis constant. The Hill coefficient is widely used as a measure of cooperativity. If the enzyme exhibits no cooperativity, then $h = 1$, and the above equation reduces to simple Michaelis–Menten eq. A Hill coefficient of greater than unity ($h > 1$) implies positive cooperativity, whereas negative cooperativity is indicated if it is less than unity ($h < 1$). A schematic of $v \rightarrow [S]$ curves for cooperative enzymes is depicted in Fig. 15.5.

For cooperative enzymes, we can show numerically that $[S]_{0.9}/[S]_{0.1}$ does not equal to 81. The $[S]_{0.9}/[S]_{0.1}$ ratio (also known as *cooperativity index, R*) itself can be used as an alternative measure of cooperativity. The $\boldsymbol{R}$ values for positively cooperative enzymes are less than 81, while for negative cooperativity, it is greater than 81. The two indices of cooperativity, namely, $\boldsymbol{h}$ and $\boldsymbol{R}$, are related to each other as shown in the box below.

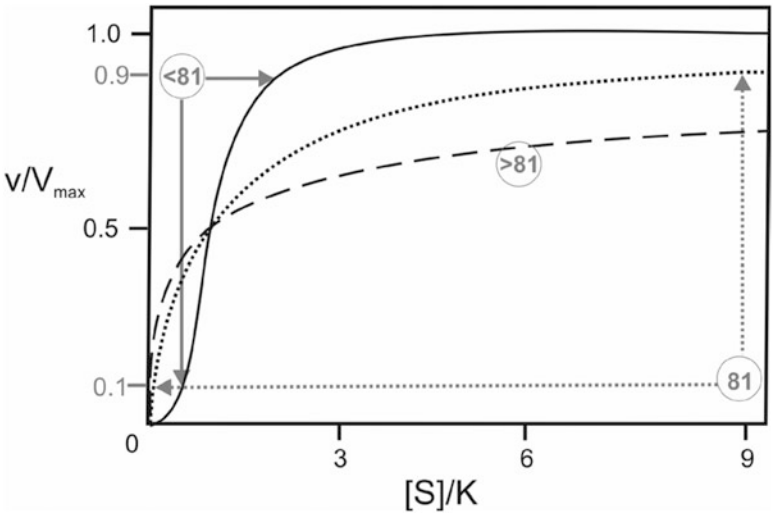

Fig. 15.5 The $v \rightarrow [S]$ curves for enzymes with different cooperativity indices. Schematic curves for positive cooperativity (solid line; $R < 81$), negative cooperativity (dashed line; $R > 81$), and Michaelis–Menten kinetics (dotted line; $R = 81$) are shown. The $[S]_{0.9}$ value for negative cooperative enzyme lies far to the right (outside the X-axis scale) and is not shown. The two axes are plotted as dimensionless quantities with fractional velocity (Y-axis) and substrate concentration relative to K (X-axis). Here K denotes K_M for the Michaelis–Menten kinetics and $K_{0.5}$ for the two cases of cooperativity

h and *r*: The Two Cooperativity Indices

The Hill equation may be rearranged as shown:

$$\frac{v}{V_{\max}} = \frac{[S]^h}{{K_{0.5}}^h + [S]^h}$$

For $v/V_{max} = 0.9$, we obtain $[S]_{0.9} = 9^{1/h} \times K_{0.5}$

Similarly, for $v/V_{max} = 0.1$, we get $[S]_{0.1} = \frac{1}{9^{1/h}} \times K_{0.5}$

Taking ratios,

$$\boldsymbol{[S]_{0.9}/[S]_{0.1} = R = 81^{1/h}}$$

We can relate the two indices by substituting different values for $\boldsymbol{h}$ in this equation. For example, when a hyperbolic (Michaelian) kinetics is described $h = 1$ gives $R = 81$.

(continued)

Type of v → [S] curve	h (or n_H)	R
Michaelis–Menten kinetics	1.0	81
Positive cooperativity ($h > 1$)	2.0	9
	4.0	3
Negative cooperativity ($h < 1$)	0.5	6560

Many cooperative enzymes also exhibit allostery and *vice versa*. Such enzymes have important roles in metabolic regulation. For instance, positive cooperativity enables them to respond with exceptional sensitivity to changes in metabolite concentration. We will revert to this topic in a later section (Chap. 37; Regulation of Enzyme Activity).

15.3 Significance of K_M, V_{max}, and k_{cat}/K_M

In the Michaelis–Menten world of the enzyme catalysis, K_M and V_{max} are the fundamental constants. As long as the equilibrium assumption is valid (i.e., $K_M \approx K_S$), K_M may be viewed as an *apparent dissociation constant* for *ES*. Otherwise it is not. It is observed that Michaelis constants recorded for most enzymes are in the range of their corresponding physiological substrate concentrations. Each metabolite (substrate) concentration in vivo is optimized by the evolutionary process for efficient functioning of cellular metabolism. This in turn drives enzyme evolution to achieve a Michaelis constant of the same magnitude. The K_M therefore provides an approximation of [*S*] in vivo. In general, biosynthetic enzymes have much lower K_M values than their corresponding catabolic counterparts (more on this may be found in Chap. 37). As a thumb rule, enzymes best operate as catalysts with [*S*] around or above their Michaelis constants – otherwise their catalytic potential is underutilized. Also, the substrate concentration region around the K_M is where the system exhibits fractional order!

The other constant V_{max} is the maximal velocity at saturating concentrations of substrate. In the classical derivation of Michaelis–Menten equation, it was equated to $k_2 \times [E_t]$. Recall that k_2 was the rate constant for the "formation and release" of product from *ES* complex (Fig. 15.1). This simplistic mechanism involved a single *ES* complex. For more general cases, with many more intermediates and steps, k_2 may be replaced by $\boldsymbol{k_{cat}}$ (and therefore, $V_{max} = k_{cat} \times [E_t]$). Regardless of the number of steps/constants it describes, k_{cat} has the units of a first-order rate constant (i.e., $time^{-1}$). This is also known as *turnover number* – and it defines the number of turnovers (catalytic cycles) – the enzyme can undergo in unit time when the enzyme

is fully saturated with substrate (also see Chap. 14). Whereas V_{max} depends on the enzyme concentration ($[E_t]$), k_{cat} does not. Therefore, k_{cat} is a fundamental property of the enzyme.

In light of the steady-state concept, a clean separation of binding (K_M) and rate of catalysis (V_{max}) may not be possible. The kinetic parameters K_M and k_{cat} (and therefore V_{max}) are algebraic aggregates of microscopic rate constants associated with many reaction steps. Recall that only in a simple mechanism (Fig. 15.1) k_2 equals k_{cat}. Otherwise, k_{cat} may also contain many more rate constants within it. Michaelis constant is a complex of at least three (and may be more if it is viewed as $(k_{-1} + k_{cat})/k_1$) rate constants and is conceptually difficult to grasp. Cleland therefore suggested that V_{max}/K_M (first-order rate constant) and V_{max} (zero-order rate constant) are the two *fundamental kinetic constants* for an enzyme-catalyzed reaction (Table 15.1). They represent apparent rate constants at very low and very high [*S*], respectively. According to this view, K_M is merely a derived constant obtained from the ratio of two rate constants (viz., $V_{max}/(V_{max}/K_M)$).

Kinetic Perfection and the Diffusion Limit Enzymes are exquisite catalysts of nature. Nevertheless, can the catalytic efficiency of an enzyme be improved further? (Albery and Knowles 1976) What then is a "perfect" enzyme? Which fundamental kinetic constant(s) provide this information? We shall now attempt to answer these interesting questions. Imagine the events during enzyme catalysis as shown in Fig. 15.6.

In this depiction, the chemical steps in a single catalytic cycle – including all the bond breaking and forming events and conformational changes – are sandwiched between two physical steps. The physical events of substrate colliding with the enzyme molecule and product dissociating from the enzyme surface are diffusion controlled. As a catalyst, an enzyme can do very little to overcome this diffusion-imposed limit. Regardless of how well an enzyme accelerates the chemical steps, *the upper bound for catalytic rate acceleration is the prevailing diffusion-limited on-rate*.

What kinetic parameter(s) of the enzyme should then be compared with diffusion-limited *on-rate* to evaluate its efficiency? At least under low [S] conditions, the Michaelis–Menten equation reduces to $v = (k_{cat}/K_M) \times [E] \times [S]$. The reaction is now effectively a bimolecular collision between free *E* and free *S*. The ratio k_{cat}/K_M

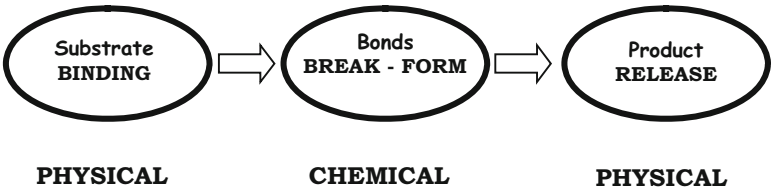

Fig. 15.6 Simplified view of events during a single cycle of enzyme catalysis. The chemical bond breaking/forming steps are sandwiched between two diffusion-controlled physical events

is therefore a good measure of enzyme's *kinetic perfection*. It has the same units as bimolecular diffusion *on-rate* constant, namely, $M^{-1} \times s^{-1}$.

$$\frac{k_{cat}}{K_M} = \frac{k_{cat}}{\frac{k_{-1}+k_{cat}}{k_1}} = \frac{k_{cat}}{k_{-1}+k_{cat}} \times k_1 \quad \left(\approx \frac{s^{-1}}{M} = M^{-1} \times s^{-1}\right)$$

Consider a situation where every collision between the enzyme and a substrate molecule is productive: that is k_{cat} (product formation rate) is much larger than k_{-1} (dissociation of *ES* back to *E* and *S*). Under these conditions, k_{cat}/K_M approximates to k_1 in the above equation. Thus the upper limit to k_{cat}/K_M is set by k_1 – the rate of formation of *ES* complex! Since k_1 is the bimolecular association rate constant between *E* and *S*, this rate cannot be faster than the rate of diffusion-controlled encounter of *E* and *S*. Notice that the units for k_1 ($M^{-1} \times s^{-1}$) are the same as those for the diffusion *on-rate* constant. Obviously, the upper bound for k_1 (and hence for k_{cat}/K_M, by the above argument) is diffusion-limited *on-rate* constant (Eisenthal et al. 2007). In this sense, k_{cat}/K_M *becomes diffusion controlled in a perfect enzyme*. For many enzymes, as noted by Cleland in 1975, the k_{cat}/K_M values approach the diffusion limit (between 10^8 and 10^9 $M^{-1} \times s^{-1}$). Triose phosphate isomerase was one of the early candidates to be characterized and termed "perfect" by this criterion. The k_{cat}/K_M ratios of superoxide dismutase and acetylcholinesterase are also between 10^8 and 10^9 $M^{-1} \times s^{-1}$. Any further gain in catalytic rate requires that the time for diffusion should decrease. The catalytic efficiencies (as k_{cat}/K_M) for a few representative enzymes are shown in Table 15.2. As can be seen, most of them have nearly attained kinetic perfection.

The concept of a kinetically perfect enzyme begs the question – *how to check whether an enzyme is diffusion limited?* We can probe this by varying the rate of diffusion through viscosity adjustments. Viscosity of aqueous solutions can be controlled by the addition of solutes like sucrose or glycerol. For instance, 30%

Table 15.2 Enzyme catalytic efficiency (k_{cat}/K_M) compared to uncatalyzed reaction rate constant (k_{uncat})

Enzyme	Catalytic efficiency[a] (k_{cat}/K_M), $M^{-1} \times s^{-1}$	Uncatalyzed rate (k_{uncat}), s^{-1}	Catalytic proficiency (k_{cat}/K_M)/k_{uncat}, M^{-1}
OMP decarboxylase	5.6×10^7	2.8×10^{-16}	2.0×10^{23}
Ketosteroid isomerase	3.0×10^8	1.7×10^{-7}	1.8×10^{15}
Triose phosphate isomerase	2.4×10^8	4.3×10^{-6}	5.6×10^{13}
Carbonic anhydrase	8.3×10^8	1.3×10^{-1}	9.2×10^8
Fumarase	1.6×10^8	–	–
Acetylcholinesterase	1.4×10^8	–	–
β-Lactamase	1.0×10^8	–	–

[a]These values are compared with corresponding bimolecular diffusion on-rate constant in water of about 10^8 and 10^9 $M^{-1} \times s^{-1}$, in water

sucrose increases the viscosity of water by a factor of 3 – the diffusion rate also is decreased by the same factor. A decrease in the k_{cat}/K_M of an enzyme with increasing medium viscosity is indicative of a diffusion-limited enzyme. If the magnitude of k_{cat}/K_M is smaller, then the bimolecular collisions occur faster and diffusion is not rate limiting. For such enzymes, k_{cat}/K_M is independent of viscosity changes. When such a study was conducted on carbonic anhydrase, indeed the hydration-dehydration rate of CO_2 decreased with increase in viscosity; the esterase function displayed by this enzyme was however unaffected. Clearly, the chemical step (and not the diffusion) is rate limiting for the nonphysiological esterase function of carbonic anhydrase (Hasinoff 1984). Experimentally sucrose or glycerol may be used to raise the medium viscosity. Such viscosogens raise the micro-viscosity of water and influence diffusion rates. Whereas polymers like Ficoll or polyethylene glycol (PEG) increase macro-viscosity (also termed bulk viscosity) but have no effect on micro-viscosity, they cannot be used to test if an enzyme is diffusion limited because they do not slow down diffusion. A word of caution with viscosity-dependence experiments: Viscosogen used and/or viscosity change may have more complex effects on the enzyme itself. Such effects (other than on k_{cat}/K_M) require more careful interpretation.

Proton diffusion on-rates in water are in the order of 10^{11} $M^{-1} \times s^{-1}$. Diffusion on-rates for bimolecular collisions in an aqueous environment are in the range of 10^{10} $M^{-1} \times s^{-1}$. When one of them is larger in size (say an enzyme), this is further diminished to 10^8–10^9 $M^{-1} \times s^{-1}$. Additional effects of local micro-viscosity and macromolecular crowding in vivo may reduce this rate further (see Chap. 38 for details). What then may be the meaning of observed k_{cat}/K_M values that are higher than the physiologically relevant diffusion-limited on-rate constant? Such abnormal high k_{cat}/K_M values could indicate one of the following:

(a) There may be something wrong with our interpretation or the estimation of second-order diffusion on-rate constant.
(b) Special enzyme features like charge guidance may be operating. For instance, strong electrostatic field gradients near the active site may cause an increase in the rate of association of superoxide dismutase with its anionic substrate (see Chap. 6, section 6.2: Contribution by electrostatics).
(c) The enzyme may be operating as a component of multienzyme aggregate. Enzyme catalyzing a step in a multienzyme sequence may directly transfer its product to the next enzyme. Bulk is minimized, and local concentrations of metabolites are enhanced by channeling. Such channeling may lead to apparently high k_{cat}/K_M values than for those enzymes acting on freely diffusible substrates. Multienzyme complexes and multifunctional polypeptides may lead to physiologically relevant regulation (Chap. 37) by confining substrates and products in a limited volume (Chap. 38).

If an enzyme reaction is diffusion rate limited, then its kinetic efficiency cannot be further improved. We see that individually both k_{cat} and K_M can take a range of values but their ratio (k_{cat}/K_M) can only reach the upper limit of diffusion rate. A

tighter binding to the substrate (lower K_M; smaller denominator) has to be offset by a smaller turnover number (lower k_{cat}; smaller numerator). Conversely, large k_{cat} values are associated with poor substrate binding by that enzyme. Recall that enzymes are devices that utilize part of the substrate binding energy to accelerate reaction rates (Chap. 6). An example of this K_M-k_{cat} *compensation*, within the bounds of diffusion-limited rates, is shown in the box below.

The Tradeoff Between k_{cat} and K_M

Two small molecules diffuse toward each other in an aqueous environment with a bimolecular rate constant of $10^{10}\ M^{-1} \times s^{-1}$. When one of the partners is large (an enzyme protein!), this rate constant is lowered to 10^8–$10^9\ M^{-1} \times s^{-1}$. Now examine the kinetic parameters for triose phosphate isomerase: K_M for glyceraldehyde-3-phosphate $= 10^{-5}$ M and $k_{cat} = 10^3\ s^{-1}$. Accordingly,

$$k_{cat}/K_M = 10^8\ M^{-1} \times s^{-1}$$

Triose phosphate isomerase therefore is almost at the "plateau of perfection."

Now consider I-*Ppo*I endonuclease. This intron-encoded endonuclease has a K_M of 4 nM for its substrate (42-bp duplex DNA). Then what should be the k_{cat} of this enzyme? Suppose if its k_{cat} value is same as that for triose phosphate isomerase (i.e., $10^3\ s^{-1}$), then we obtain

$$k_{cat}/K_M = \left(10^3\ s^{-1}\right)/\left(4 \times 10^{-9}\ M\right) = 2.5 \times 10^{11}\ M^{-1} \times s^{-1}$$

Such a value is beyond the limit set by diffusion-limited on-rate and hence is not feasible. A realistic k_{cat} for this enzyme at best should therefore be $4\ s^{-1}$.

Clearly a smaller value of K_M in the denominator (of k_{cat}/K_M) constrains the upper limit that k_{cat} can approach. Both the enzymes will have reached *kinetic perfection* because their k_{cat}/K_M values are close to the diffusion limit. It is just that triose phosphate isomerase has evolved for a higher k_{cat} ($10^3\ s^{-1}$) but with a reasonable K_M (10 μM), while I-*Ppo*I endonuclease is set to a lower k_{cat} but with tighter interaction with substrate (a lower K_M of 4 nM). In fact, this k_{cat}-K_M compensation forms the basis for the functional existence of isozymes (discussed later in this chapter).

Other Interpretations of k_{cat}/K_M

Specificity, besides excellent catalysis, is hallmark of an enzyme catalyst (Chap. 5). How then should we define specificity of an enzyme? Without question, the ratio k_{cat}/K_M provides insight into this aspect of an enzyme. This ratio has been referred to as the *specificity constant* – because it describes the relative velocities of two substrates

Table 15.3 Specificity constants for competing fumarase substrates

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1} \times s^{-1}$) (specificity constant)
Fumarate	5	800	160
Fluorofumarate	27	2667	99
Chlorofumarate	110	22	0.20
Bromofumarate	110	2.8	0.025

competing for a single enzyme (Koshland Jr 2002). This competition between S_1 and S_2 is given by

$$\frac{v_1}{v_2} = \left(\frac{k_{cat1}}{K_{M1}}[S_1]\right) / \left(\frac{k_{cat2}}{K_{M2}}[S_2]\right)$$

Specificity constant, therefore, is the parameter that determines the ratio of rates when competing substrates are vying for the same enzyme. Thus k_{cat}/K_M expresses the ability of an enzyme to discriminate in favor of any one substrate over the others. It can rank structurally similar substrates with respect to catalytic power of an enzyme. This is illustrated by the kinetic data (Table 15.3) for fumarase and its substrates (Teipel et al. 1968).

Since its k_{cat} (zero-order rate constant) is higher (compared to other fumarates) at saturating concentrations, fluorofumarate appears to be a better substrate. And at lower concentrations (k_{cat}/K_M; first-order rate constant condition), fumarate is better. However, when the two are present together, k_{cat}/K_M best expresses the ability of fumarase to discriminate in favor of fumarate. In a similar analysis, the specificity constants for peptide bond hydrolysis by chymotrypsin are as follows: R group of Gly < Val < Tyr.

The k_{cat}/K_M goes up as the enzyme shows higher affinity for the substrate and/or higher catalytic rates. In general, higher the k_{cat}/K_M better is the enzymatic performance. This is true for one enzyme acting on many substrates (for instance, fumarase data in Table 15.3 above) but is also valid when many mutant forms of the same enzyme, acting on a single substrate, are compared. Therefore, Koshland preferred the term *performance constant* to describe k_{cat}/K_M.

The catalytic power of an enzyme was related to the corresponding uncatalyzed reaction rate by Radzicka and Wolfenden (1995). The second-order rate constant for enzyme action on its substrate (i.e., k_{cat}/K_M) may be compared to the corresponding uncatalyzed rate. *Catalytic proficiency* defined this way measures an enzyme's ability to lower the activation energy barrier ($\Delta G^{\neq}$) for that reaction (Chap. 5). The spontaneous, uncatalyzed rate constants (k_{uncat} values) vary over 15 orders of magnitude (Table 15.2). The k_{cat}/K_M values for the corresponding enzyme reactions however fall within a narrow range of three orders. And none cross the ceiling of $\approx 10^9\ M^{-1} \times s^{-1}$ – the diffusion rate limit!

There is one other descriptor for k_{cat}/K_M. According to Northrop, k_{cat}/K_M (or V_{max}/K_M) actually provides a measure of the rate of substrate capture – by the free enzyme – into a productive complex (Northrop 1998). Thus $k_{capture}$ *is the rate of substrate capture* into *ES* complex (Fig. 15.1). Given this, k_{cat} (or V_{max}) may be viewed as a measure of the rate of release of product ($k_{release}$), from the captured complex. The ratio $k_{release}/k_{capture}$ now defines K_M as a derived parameter. The perception of k_{cat}/K_M as $k_{capture}$ (and its physiological relevance!) is beautifully demonstrated by antibiotic resistance in bacteria (Radika and Northrop 1984). When present, aminoglycoside acetyltransferase confers antibiotic resistance to the host bacterium. This enzyme inactivates aminoglycoside antibiotics by acetylating them. A correlation between MIC (minimal inhibitory concentration) and the kinetic characteristics of this enzyme (i.e., V_{max}, K_M, and V_{max}/K_M) was attempted. No robust relationship exists between MICs of different aminoglycosides with their corresponding V_{max} or K_M values. But a very tight correlation ($R^2 = 0.995$) is seen between their MIC values and V_{max}/K_M for the enzyme. Executing a successful capture (meaning a large $k_{capture}$) is vitally important for antibiotic resistance and hence bacterial survival. Once captured, the fate of the antibiotic is sealed – it does not matter how long it actually takes for the enzyme to form and release the product. Rates of product release ($k_{release}$) are not very impressive for these enzymes.

15.4 Haldane Relationship: Equilibrium Constant Meets Kinetic Constants

The classic form of Michaelis–Menten equation (earlier in this chapter) was derived for a reaction proceeding in a single direction ($S \rightarrow P$). For reasons practical and otherwise, rate of $P \rightarrow S$ was set to zero. Many reactions of metabolism however are reversible, and significant amounts of both substrate and product are present at any given time. Therefore it is important to include the reverse reaction as well. Although a bit more complicated, it is possible to derive the rate equation for a reversible reaction. Figure 15.1 describes the simplest case where k_{-2} is finite and $[P] \neq 0$. For a single *ES* complex example of the reversible enzyme reaction ($S \rightleftarrows P$; Fig. 15.1), we can derive the following Michaelis–Menten equation (for the actual derivation, see Chap. 16):

$$v = \frac{\frac{V_{maxf}[S]}{K_{MS}} - \frac{V_{maxr}[P]}{K_{MP}}}{1 + \frac{[S]}{K_{MS}}} + \frac{[P]}{K_{MP}}$$

This equation is a more general form of the Michaelis–Menten equation where both forward and reverse reaction rates are considered. It collapses to the simple form (for $S \rightarrow P$ reaction) by putting $[P] = 0$ in this equation. The equation is symmetric with respect to $S \rightarrow P$ and $P \rightarrow S$. If we put $[S] = 0$, then the equation collapses to the classic form of Michaelis–Menten equation for the reverse direction.

However, when both S and P are present and when the system has reached equilibrium (see Chap. 10), the net velocity is zero. Putting $v = (v_f - v_r) = 0$ in the general rate equation above and simplifying,

$$v = \frac{\frac{V_{maxf}[S]}{K_{MS}} - \frac{V_{maxr}[P]}{K_{MP}}}{1 + \frac{[S]}{K_{MS}}} + \frac{[P]}{K_{MP}} = 0$$

Therefore,

$$\frac{V_{maxf}[S]}{K_{MS}} - \frac{V_{maxr}[P]}{K_{MP}} = 0$$

and

$$\frac{V_{maxf}[S]}{K_{MS}} = \frac{V_{maxr}[P]}{K_{MP}}$$

Substituting the corresponding equilibrium concentrations and rearranging, we get

$$\frac{V_{maxf} \times K_{MP}}{V_{maxr} \times K_{MS}} = \frac{k_{catf} \times K_{MP}}{k_{catr} \times K_{MS}} = \frac{[P]_{eq}}{[S]_{eq}} = K_{eq} \quad \textbf{Haldane relationship}$$

This equation relates enzyme kinetic constants to the overall equilibrium constant of that reaction. This relationship was first shown by JBS Haldane and hence the name. The first-order rate constants for the forward (V_{maxf}/K_{MS}) and reverse (V_{maxr}/K_{MP}) direction of a reversible enzyme-catalyzed reaction are not independent. But they are related to the equilibrium constant (K_{eq}) of the overall reaction. The following conclusions may be drawn from the Haldane relationship:

(a) Both V_{maxf} and V_{maxr} may be written as their corresponding $k_{cat} \times [E_t]$ terms. Further, V_{max} depends on the enzyme concentration ($[E_t]$) and k_{cat} does not. Since the $[E_t]$ appears both in the numerator and the denominator, *the equilibrium constant (K_{eq}) is independent of enzyme concentration*.
(b) Haldane equation can be conveniently written as

$$\left(\frac{k_{catf}}{K_{MS}}\right) / \left(\frac{k_{catr}}{K_{MP}}\right) = K_{eq}$$

The equilibrium constant is then nothing but the ratio of k_{cat}/K_M values of the forward and reverse reactions. Reaction equilibrium constant is a thermodynamic constant and cannot change merely because an enzyme is present. Therefore, K_{eq} *puts a constraint on what values the enzyme kinetic constants (both* k_{cat} *and* K_M*) could take*. This is yet another case where k_{cat}/K_M for an enzyme assumes importance.

Kinetic Feasibility of Isozymes Effectively there are two constraints operating on the kinetic constants of an enzyme catalyst. One is the diffusion rate barrier (on k_{cat}/K_M value) and the other is thermodynamic – set by the Haldane relationship. The reaction K_{eq} dictates only that the ratio $(V_{maxf}/K_{MS})/(V_{maxr}/K_{MP})$ remains a constant. Within these constraints, many numerical solutions are possible – giving the same K_{eq} but with different k_{cat} and K_M values. This is very interesting because enzymes with different V_{max} (or k_{cat}) and K_M values could be constructed for the same reaction – without violating the two constraints mentioned above. One such example is shown in the box below.

Haldane Relationship and Isozymes

Consider a reversible reaction "$S \rightleftarrows P$" catalyzed by two distinct enzymes, namely, Enz-I and Enz-II. Suppose their kinetic constants are as shown:

Enz-I:	$V_{maxf} = V_{maxr} = 100$	$K_{MS} = 5$ mM	$V_{maxf}/K_{MS} = 20$
		$K_{MP} = 5$ mM	$V_{maxr}/K_{MP} = 20$
Enz-II:	$V_{maxf} = V_{maxr} = 100$	$K_{MS} = 100$ mM	$V_{maxf}/K_{MS} = 1$
		$K_{MP} = ??$	

We infer the following from this data:

(a) Enz-I is relatively more efficient in the forward direction. Its V_{maxf}/K_{MS} is 20 times that of Enz-II.

(b) The K_{eq} for this reaction ($S \rightleftarrows P$) may be calculated from the given data for Enz-I.

$$\frac{V_{maxf} \times K_{MP}}{V_{maxr} \times K_{MS}} = \frac{100 \times 5}{100 \times 5} = 1$$

(c) We now predict that the K_{MP} for Enz-II should be 100 mM! Why so? Because it is the only numerical solution that gives an equilibrium constant of unity (i.e., $K_{eq} = 1$).

(continued)

$$K_{eq} = \frac{V_{maxf} \times K_{MP}}{V_{maxr} \times K_{MS}} = \frac{100 \times 100}{100 \times 100} = 1$$

Any other value (including a K_{MP} of 5 mM, like for Enz-I) will yield a different K_{eq} – recall that reaction equilibrium constant is a thermodynamic parameter that the catalyst cannot tinker with.

Multiple catalytic designs for the same reaction are possible – this is the basis for the existence of isozymes in nature. Isozymes will have compensated for differences in their forward kinetic constants (V_{maxf} and/or K_{MS}) by suitable adjustments in their reverse kinetic constants (V_{maxr} and/or K_{MP}) – thereby resulting in an identical K_{eq} value. The isozyme with a lower V_{maxf}/K_{MS} could either have an appropriately lowered V_{maxr}, an elevated K_{MP} or both.

A consequence of multiple kinetic solutions for the same reaction is that isoforms of an enzyme catalyst are possible. Nature has exploited these solutions in the form of *isozymes* (or isoenzymes). Isozymes are multiple molecular forms that catalyze the same chemical reaction. Among others, lactate dehydrogenases (the heart and the muscle isoforms) and alcohol dehydrogenase (yeast ADH-I and ADH-II) are excellent examples. Bacteria elaborate two distinct isoforms of threonine deaminase for biosynthesis (with higher affinity for Thr; low K_M) and catabolism (with lower affinity for Thr; high K_M). Isozymes play critical roles in cellular metabolic regulation. We will have more to say on this later (in Chap. 37).

Although Haldane relationship places limits on the kinetic constants, a wide range of enzyme kinetic behavior is still allowed. As a result, we can expect an enzyme evolved to be more effective catalyst for one direction of a reaction than the other. Indeed such *one-way enzymes* are possible. For instance, limiting rate of the forward reaction catalyzed by methionine adenosyltransferase is about 10^5 times greater than its reverse reaction rate. The enzyme efficiency can be improved for one direction, at the expense of the other, by optimizing k_{cat}/K_M values to suit prevailing concentrations of substrate and product. One-way enzymes also make physiological sense – they may never be required to catalyze the reaction in the reverse direction in vivo. There may be no evolutionary pressure to achieve catalytic perfection in that direction! If the active site is strictly complementary to the transition state, then the enzyme will be an optimized catalyst for both directions. Efficiency in one direction could however be preferentially improved by evolving an active site that binds either S or P relatively better, with nearly the same transition state. Finally, since $V_{maxf} = k_{catf} \times [E_t]$, any unfavorable k_{catf} changes (arising out of thermodynamic constraints) during catalyst design/evolution can be compensated by the system. A cell can maintain the desired V_{maxf} by increasing $[E_t]$ (increased cellular abundance!) despite having a lower k_{catf}. Therefore, the cellular concentrations of various isozymes (and enzymes in general!) are not necessarily maintained at the same level (Futcher et al. 1999).

15.5 Use and Misuse of Michaelis–Menten Equation

Apart from its historical importance, Michaelis–Menten equation undoubtedly remains a very important tool in enzyme kinetics. It is the first useful approximation for any new enzyme to be studied. One must however be confident that the Michaelis–Menten equation is obeyed by that enzyme – for not all enzymes adhere to Michaelis–Menten kinetics. Careful analysis of kinetic measurements may indicate possible deviations, such as substrate inhibition. There is no need to force Michaelis–Menten equation on every enzyme – after all data dictates!

At some level all scientific representations are approximations. In this sense, Michaelis–Menten equation is an outcome of explicit postulates and assumptions made in its derivation. The most elementary consideration is that all the $v \rightarrow [S]$ kinetic data used are initial velocities. The general applicability of the Michaelis–Menten model also requires that $[S_t] \approx [S]$ and that $[E_t] << [S_t]$. If any one or more of these stipulated conditions are not met, then the model is unsuitable for use. Other relevant modifications to the Michaelis–Menten equation become necessary before adopting such a kinetic model.

Both V_{max} (which is $k_{cat} \times [E_t]$) and K_M are intrinsic constants for a given enzyme. The V_{max} in principle may be adjusted by varying the total enzyme present, whereas k_{cat} is unique for that catalyst. The Michaelis constant (the K_M) is a constant defined according to the Michaelis–Menten model (and hyperbolic kinetics). Any interpretation of K_M becomes irrelevant the moment Michaelis–Menten model is not obeyed. Even within these confines, the K_M provides only an apparent measure of substrate affinity. That implies "$K_M = K_S$" is not always true (especially when k_{cat} is not negligibly small in comparison with k_{-1}) and should not be taken for granted without sufficient evidence.

Despite the divergent views on interpreting k_{cat}/K_M as "specificity constant," "performance constant" (Koshland), "catalytic proficiency" (Wolfenden), or "rate of substrate capture" (Northrop), we note that k_{cat}/K_M is of great practical significance. When the magnitude of k_{cat}/K_M is comparable to the diffusion-limited on-rate constant, the steady-state approximation operates. For all lower values (i.e., $k_{cat}/K_M < k_1$), the equilibrium mechanism is more appropriate. Lastly, catalytic perfection may not be the only constraint worked on the enzyme by evolution! Regulation and cost efficiency may be two others, at the least.

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Suggested Reading

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