Preprint

Visual Interpretation of the Meaning of $k_{\text{cat}}/K_{\text{M}}$ in Enzyme Kinetics

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RUNNING TITLE: Visual Interpretation of the Meaning of $k_{\text{cat}}/K_{\text{M}}$

Abstract

k_{cat} and k_{cat}/K_M are the two fundamental kinetic parameters in enzyme kinetics. k_{cat} is the first-order rate constant that determines the reaction rate when the enzyme is fully occupied at a saturating concentration of the substrate. k_{cat}/K_M is the second-order rate constant that determines the reaction rate when the enzyme is mostly free at a very low concentration of the substrate. Both parameters provide critical information on how the enzyme lowers the energy barriers along the reaction pathway for catalysis. However, it is surprising how often k_{cat}/K_M is used inappropriately as a composite parameter derived by dividing k_{cat} with K_M to assess both catalytic power and affinity to the substrate of the enzyme. The main challenge in explaining the true meaning of k_{cat}/K_M is the difficulty to demonstrate how the reaction energetics of enzyme catalysis determines k_{cat}/K_M in a simple way. Here, I report a step-by-step demonstration on how to visualize the meaning of k_{cat}/K_M on the reaction energy diagram. By using the reciprocal form of the expression of k_{cat}/K_M with the elementary rate constants in kinetic models, I show that k_{cat}/K_M is a harmonic sum of several kinetic terms that correspond to the heights of the transition states relative to the free enzyme. Then, I demonstrate that the height of the highest transition state has the dominant influence on k_{cat}/K_M , i. e. the step with the highest transition state is the limiting step for k_{cat}/K_M . The visualization of the meaning of k_{cat}/K_M on the reaction energy diagram offers an intuitive way to understand all the known properties of k_{cat}/K_M , including the Haldane relationship.

Keywords

enzyme kinetics; kinetic parameters; reaction energy diagram; rate-limiting step; kinetic model; rate equation;

Footnotes

1. The traditional form of the Michaelis-Menten equation was written with $V_{\max}$, instead of $k_{\text{cat}}E_t$, as:

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

The main reason of the popular use of $V_{\max}$ instead of k_{cat} in the past was that the concentration of the total active enzyme (E_t) was unknown or inaccurate in many cases. For the same reason, V/K was more popularly used than k_{cat}/K_M . While k_{cat} and k_{cat}/K_M are kinetic parameters characteristic for an enzyme and a substrate, $V_{\max}$ and V/K are kinetic parameters determined with an enzyme at an arbitrary concentration. As protein purification techniques have advanced and the analytical methods for protein concentration determination have improved, $V_{\max}$ and V/K are often replaced with k_{cat} and k_{cat}/K_M .

2. Many biochemistry textbooks use an even simpler kinetic model for the derivation of the Michaelis–Menten equation:

However, this kinetic model is not suitable in dissecting the meanings of k_{cat} and k_{cat}/K_M rigorously because the chemical conversion step ($\text{ES} \rightleftharpoons \text{EP}$) and the product release step ($\text{EP} \rightarrow \text{E} + \text{P}$) are not separated, and k_{cat} is determined by a single elementary rate constant (k_3)! The separation of the two steps is indeed necessary because the product release, not the chemical conversion, limits the overall turnover in many enzyme-catalyzed reactions (1).

3. The rate equation can be derived either with the King–Altman method (2) or with the net rate constant method (3). Especially, the inclusion of the single irreversible step for the product release in Model 1 allows us to use the net rate constant method for the derivation of the rate equation.
4. The expression for K_M for Model 1 is even more complex than those for k_{cat} or k_{cat}/K_M :

$$K_M = \frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1(k_3 + k_4 + k_5)}$$

5. As the name implies, the pseudo-first-order rate constant is not a true rate constants; when [S] varies, the constant varies accordingly. In enzyme kinetics, however, we treat the pseudo-first-order rate constants as same as any other first-order rate constants because typically we deal with the kinetics of enzymatic catalysis at a fixed concentration of [S]. Treating [S] as a constant is also a common practice in experimental determination of the initial velocity (v_0); the initial velocity should be determined within a timeframe in which [S] does not change significantly.
6. We can demonstrate the mathematical relationship between the heights of the transition states and k_1k_3/k_2 and $k_1k_3k_5/k_2k_4$, using the property that the barrier height in the reaction energy diagram is linearly proportional to $\ln(1/k)$.

$$\ln \frac{k_2}{k_1k_3[S]} = \ln k_2 - \ln k_1[S] - \ln k_3 = \ln \frac{1}{k_1[S]} - \ln \frac{1}{k_2} + \ln \frac{1}{k_3}$$

The three terms on the right side are linearly proportional to the barrier heights corresponding to $k_1[S]$, k_2 , and k_3 , respectively. By subtracting and adding the arrow lengths in the reaction energy diagram in Figure 2, one can visually verify that k_1k_3/k_2 corresponds to the height of the transition state for the chemical conversion step ($ES \rightarrow EP$) relative $E + S$. Using the same approach, we can demonstrate that $k_1k_3k_5/k_2k_4$ corresponds to the height of the transition state for the product release step ($EP \rightarrow E+P$) relative $E + S$.

7. ‘Rate-limiting’ is not a suitable word for k_{cat}/K_M because k_{cat}/K_M is not a rate but a rate constant. I still use ‘rate-limiting’ in this manuscript simply following the convention.
8. The rate-limiting step for k_{cat}/K_M is not necessarily the rate-limiting step for k_{cat} . At a saturating concentration of the substrate, the amount of the free enzyme (E) is negligible. i.e. the bound states of the enzyme (ES and EP in Model 1) are highly populated, and the highest transition state may not have the dominant influence on k_{cat} . For example, the substrate association step cannot not affect k_{cat} , even when its transition state is the highest. Moreover, the application of the concept of the rate-limiting step to k_{cat} is complicated and open to question (4).

9. Practically, it is quite unlikely to raise the energy of a trough without affecting the height of neighboring transition states because the structural similarity exists between the intermediate and the transition states. The treatment of selectively raising the energy of a trough here is only for a conceptual assessment of the property of k_{cat}/K_M .
10. One can think of an extreme case in which the transition states for the substrate association step and for the product release step have the same height, and the equilibrium constant of the reaction is 1. Even in this extreme case, k_{cat}/K_M for both forward and reverse reactions can reach up to only half of the diffusion limit, because two transition states have the same contribution to k_{cat}/K_M (Eq. 9 and Table 1).

Introduction

When an enzyme catalyzes the conversion of a substrate to a product, the dependence of the reaction rate (v) on the substrate concentration ($[S]$) follows the following simple rate law:¹

$$v = \frac{k_{\text{cat}}[S]}{K_M + [S]} E_t \quad (1)$$

which is known as the Michaelis–Menten equation. When the substrate concentration is much greater than K_M , i.e. $[S] \gg K_M$, Eq. 1 is simplified as

$$v = k_{\text{cat}} E_t \quad (2)$$

Under this condition, the reaction is first-order; the reaction rate is dependent only on the total enzyme concentration, not on the substrate concentration (Figure 1). Therefore, k_{cat} is a first-order rate constant with the unit of s^{-1} and frequently called the ‘turnover number’, the number of substrate molecules that one enzyme can catalyze per unit time. Also, $k_{\text{cat}} E_t$ is the maximum reaction rate (V_{max}) that the enzyme can achieve under the given experimental condition. When the substrate concentration is much less than K_M , i.e. $[S] \ll K_M$, Eq. 1 is simplified as

$$v = \frac{k_{\text{cat}}}{K_M} [S] E_t \quad (3)$$

Under this condition, the reaction is second-order; the reaction rate is proportional to both substrate concentration and enzyme concentration (Figure 1). Therefore, k_{cat}/K_M is a second-order kinetic constant with the unit of $\text{M}^{-1}\text{s}^{-1}$. k_{cat}/K_M is frequently called the ‘specificity number’ because k_{cat}/K_M varies greatly with different substrates. The Michaelis constant (K_M) is just the ratio of k_{cat} to k_{cat}/K_M and corresponds to the concentration of the substrate at which half of the enzyme is saturated. Enzymologists commonly use the two fundamental kinetic parameters, k_{cat} and k_{cat}/K_M , to investigate the mechanism of enzymatic catalysis (1, 5, 6). Most of all, k_{cat} and k_{cat}/K_M allow them to prove the nature of the rate-limiting step in the enzyme-catalyzed reaction. Frequently, the changes in k_{cat} and k_{cat}/K_M are monitored in varying assay conditions (pH, temperature, salts, viscosity, or the presence of an inhibitor or an activator), with different substrates, or with variants of enzymes to dissect the detailed mechanism of the enzymatic catalysis. Being the ratio of the two fundamental kinetic constants, the effects of the change in the experimental condition on K_M is quite complex and usually do not provide any interpretable or meaningful information on enzymatic catalysis (1, 5).

While it has been well accepted among enzymologists that k_{cat} and k_{cat}/K_M are the two fundamental kinetic parameters for enzymatic catalysis, a general misunderstanding of k_{cat}/K_M is still prevalent in literature and even in biochemistry textbooks. Frequently, k_{cat} and K_M are considered as the two primary kinetic parameters one can obtain from the Michaelis-Menten equation, and k_{cat}/K_M is considered as the ratio of k_{cat} to K_M , which is mathematically true, but tremendously misleading. Considering k_{cat}/K_M as a composite parameter greatly hinders the understanding of the true meaning of k_{cat}/K_M and the proper interpretation of k_{cat}/K_M in studying enzymatic catalysis. The reason for the confusion of k_{cat}/K_M as a composite parameter is partly because the symbol of the kinetic parameter has the form of the ratio of k_{cat} to K_M . To avoid this issue, a new symbol k_{cap} (capture constant) has been proposed to replace k_{cat}/K_M (5), but unfortunately, the new notation has not been widely accepted. However, the major challenge regarding k_{cat}/K_M is the difficulty to figure out the relationship between k_{cat}/K_M and the elementary rate constants from the expression of k_{cat}/K_M (see Eq. 8 below as an example). It has been shown from the mathematical expression that k_{cat}/K_M is the ‘net’ rate constant for the substrate

association step ($E + S \rightarrow ES$) (3) or the apparent rate constant for the ‘capture’ of the substrate (5), reporting the kinetics of the formation of the enzyme-substrate complex (ES) that eventually “goes on to produce the product” (1) or “are destined to yield the product” (7). However, to a non-expert in enzyme kinetics, these explanations are not so easy to understand either.

Here I report a simpler way to explain the meaning of k_{cat}/K_M visually on the reaction energy diagram using the reciprocal expressions of k_{cat}/K_M . First, using the reciprocal expressions of k_{cat}/K_M , I show that k_{cat}/K_M is a harmonic sum of several kinetic terms, i.e. the reciprocal form of k_{cat}/K_M is the sum of the reciprocal forms of a group of kinetic terms. This approach to express k_{cat}/K_M as a harmonic sum of several kinetic terms has been used in literature (8, 9), but its value in deciphering the meaning of k_{cat}/K_M has not been fully explored. Next, I examine the meaning of each kinetic term comprising k_{cat}/K_M using the reaction energy diagram and explain that each term actually corresponds to the height of the barrier corresponding to an individual step in the kinetic model. This analysis clearly shows that k_{cat}/K_M is determined by the heights of the transition states relative to $E + S$ and influenced (or limited) dominantly by the highest transition state. Using this approach, I also demonstrate that the previously known properties of k_{cat}/K_M , including the Haldane relationship, can be explained based on reaction energetics.

Reciprocal Expression for k_{cat}/K_M

Here is a minimal, but realistic kinetic model for an enzyme-catalyzed reaction:²

(Model 1)

For simplicity, I assume that the reaction has only one substrate and one product. This model contains five rate constants. While k_2 , k_3 , k_4 , and k_5 are first-order rate constants, k_1 is a second-order rate constant for the substrate association. The product-release step is reversible in many enzyme-catalyzed reactions;

however, we usually make the kinetic studies simpler by setting up the reaction only with the substrate and the enzyme (without the product), i.e. $[P] = 0$. Under this condition, the reverse reaction ($P \rightarrow S$) is negligible, and only the forward reaction ($S \rightarrow P$) determines the overall reaction rate, which allows us to determine k_{cat} and k_{cat}/K_M for the forward reaction without any unnecessary complexity. Moreover, the inclusion of the irreversible step makes the derivation of the rate equation much easier. I will revisit later the case of enzymes that work under the condition that the substrate and the product are in equilibrium.

By using the steady-state approximation, we derive the rate equation for Model 1 as:³

$$v = \frac{k_1 k_3 k_5 [S]}{k_2 k_4 + k_2 k_5 + k_3 k_5 + k_1 (k_3 + k_4 + k_5) [S]} E_t \quad (4)$$

When the substrate concentration is much greater than K_M , i.e. $[S] \gg K_M$, the rate is determined by k_{cat} (Eq. 2) and Eq. 4 is simplified as:

$$v = k_{\text{cat}} E_t = \frac{k_3 k_5}{k_3 + k_4 + k_5} E_t \quad (5)$$

Therefore,

$$k_{\text{cat}} = \frac{k_3 k_5}{k_3 + k_4 + k_5} \quad (6)$$

When the substrate concentration is much less than K_M , i.e. $[S] \ll K_M$, the rate is determined by k_{cat}/K_M (Eq. 3), and Eq. 4 is simplified as

$$v = k_{\text{cat}}/K_{\text{M}}[S]E_{\text{t}} = \frac{k_1 k_3 k_5}{k_2 k_4 + k_2 k_5 + k_3 k_5} [S]E_{\text{t}} \quad (7)$$

Therefore,

$$k_{\text{cat}}/K_{\text{M}} = \frac{k_1 k_3 k_5}{k_2 k_4 + k_2 k_5 + k_3 k_5} \quad (8)$$

Even with this minimal kinetic model, it is difficult to figure out directly from Eq. 8 how each step in the kinetic model contributes to $k_{\text{cat}}/K_{\text{M}}$. The difficulty to decipher the mathematical expression of $k_{\text{cat}}/K_{\text{M}}$ like Eq. 8 is a potential factor causing the prevalent misconception of $k_{\text{cat}}/K_{\text{M}}$ as a composite kinetic parameter (a ratio of k_{cat} to K_{M}) rather than a fundamental kinetic parameter.⁴

The reciprocal form of Eq. 8 offers us a much simpler way to demonstrate the meaning of $k_{\text{cat}}/K_{\text{M}}$.

$$\frac{1}{k_{\text{cat}}/K_{\text{M}}} = \frac{1}{k_1} + \frac{k_2}{k_1 k_3} + \frac{k_2 k_4}{k_1 k_3 k_5} = \frac{1}{k_1} + \frac{1}{\frac{k_1 k_3}{k_2}} + \frac{1}{\frac{k_1 k_3 k_5}{k_2 k_4}} \quad (9)$$

According to Eq. 9, $k_{\text{cat}}/K_{\text{M}}$ is actually the harmonic sum (the reciprocal of the sum of the reciprocals of each member) of three kinetic terms: k_1 , $k_1 k_3/k_2$, and $k_1 k_3 k_5/k_2 k_4$. This expression shows that $k_{\text{cat}}/K_{\text{M}}$ is ‘mathematically’ analogous to the total resistance of a parallel circuit composed of resistance of k_1 , $k_1 k_3/k_2$, and $k_1 k_3 k_5/k_2 k_4$. Note that in reality the rate constant is opposite to the resistance; the greater the rate constant is, the faster the reaction is. According to the mathematical relationship shown in Eq. 9, the

smaller value of k_1 , k_1k_3/k_2 , and $k_1k_3k_5/k_2k_4$ has the greater influence on k_{cat}/K_M . Moreover, k_{cat}/K_M is always smaller than the smallest of the three terms and converges to the smallest term when the other two terms are significantly greater than the smallest term (Table 1). It is analogous to the fact that in a parallel electric circuit the current flows most through the path with the smallest resistance, which has the dominant influence on the overall resistance.

Now let's figure out the meanings of k_1 , k_1k_3/k_2 , and $k_1k_3k_5/k_2k_4$. The meaning of k_1 is obvious; by definition, k_1 is the rate constant for the formation of the ES complex. Deciphering the meanings of k_1k_3/k_2 and $k_1k_3k_5/k_2k_4$ requires the consideration of the reaction energetics. The reaction energy diagram (Figure 2) shows the relative height of the barrier for each step in Model 1. For a first-order rate constant, the corresponding barrier is obviously $\Delta G^\ddagger$ of the transition-state theory, which is linearly proportional to $\ln(1/k)$; thus, the smaller the rate constant, the higher the barrier is. To include k_1 , a second-order rate constant, in the reaction energy diagram with first-order rate constants together, one needs to convert k_1 to a pseudo-first-order rate constant by multiplying by $[S]$.⁵ Therefore, the first barrier from E+S in the reaction energy diagram is determined by $k_1[S]$. We make the level of E + S much lower than ES or EP in the reaction energy diagrams because k_{cat}/K_M determines the kinetics when the substrate concentration is low, i.e. most of the enzyme is in the free form (E). In the reaction energy diagram, k_1 , k_1k_3/k_2 and $k_1k_3k_5/k_2k_4$ have clear physical meanings. As shown in Figure 2, k_1 , k_1k_3/k_2 and $k_1k_3k_5/k_2k_4$, when multiplied by $[S]$, correspond to the heights of the transition states relative to E + S for the substrate association step ($E + S \rightarrow ES$), the chemical conversion step ($ES \rightarrow EP$), and the product release step ($EP \rightarrow E+P$), respectively.⁶ Therefore, the reciprocal expression of k_{cat}/K_M (Eq. 9) shows that k_{cat}/K_M is determined by the heights of the three transition states relative to E + S.

Rate-limiting step for k_{cat}/K_M

Figure 2 shows three versions of the reaction energy diagrams for Model 1. The substrate association step ($E + S \rightarrow ES$), the chemical conversion step ($ES \rightarrow EP$), and the product release step ($EP \rightarrow E+P$) has the highest transition state in Figures 2A, 2B, and 2C, respectively. Because the height of the transition state

is linearly proportional to $\ln(1/k)$, the least of k_1 , k_1k_3/k_2 and $k_1k_3k_5/k_2k_4$ has the highest transition state and also the greatest influence on k_{cat}/K_M . In other words, the step with the highest transition state in each reaction energy diagram in Figure 2 is the rate-limiting step for k_{cat}/K_M .⁷ In reverse, measuring of k_{cat}/K_M of an enzyme-catalyzed reaction is a way to probe the highest transition state of the reaction. Note that the relative heights of the transition states do not change when the substrate concentration is varied. When the substrate concentration is increased, only the level of E + S on the reaction energy diagrams moves up, and the relative heights of the transition states remain same. Therefore, information obtained from k_{cat}/K_M on the relative heights of the transition states is still valid at any substrate concentration.⁸

When the transition state for the substrate association step ($E + S \rightarrow ES$) is the highest (Figure 2A), k_{cat}/K_M is determined mostly by the kinetics of the substrate association ($k_{\text{cat}}/K_M \sim k_1$). When the enzyme catalyzes the chemical conversion efficiently, the substrate association step frequently becomes the rate-limiting step for k_{cat}/K_M . Because the substrate association cannot be faster than the collision between the enzyme and the substrate by diffusion, the kinetics of diffusion is the ultimate physical limit to k_{cat}/K_M . When k_{cat}/K_M reaches to this physical limit, k_{cat}/K_M is said to be diffusion-controlled (10). Depending on the size of the substrate, k_{cat}/K_M can reach up to 10^8 – $10^9 \text{ M}^{-1}\text{s}^{-1}$. Some enzymes, such as fumarase (10) and triose phosphate isomerase (11), are known to have k_{cat}/K_M values that approach this limit. The substrate association is normally much slower than diffusion because it is not likely that every collision leads to the formation of the enzyme–substrate complex capable for catalysis (ES) (12, 13).

When the transition state for the chemical conversion ($ES \rightarrow EP$) or the product release ($EP \rightarrow E + P$) is the highest (Figures 2BC), k_{cat}/K_M is determined mostly by k_1k_3/k_2 or $k_1k_3k_5/k_2k_4$, respectively. As k_2/k_1 is the dissociation equilibrium constant for the ES complex (K_S), k_{cat}/K_M in Figure 2B is approximated as k_3/K_S . In this case, k_{cat}/K_M is indeed the measure of both catalytic power (k_3) and affinity to the substrate (K_S). Even though this relationship is valid only when the transition state for the chemical conversion is the highest, it is so commonly used as the general meaning of k_{cat}/K_M . Because enzymes in nature are highly evolved to catalyze the chemical conversion, it is somewhat rare that the transition state for the chemical conversion is the highest with their physiological substrates. Therefore, the interpretation

of k_{cat}/K_M as the measure of both catalytic power (k_3) and affinity to the substrate (K_S) should be reserved for poor substrates or catalytically impaired variants of enzymes. Also, as commonly as k_{cat}/K_M is limited by the substrate association, k_{cat}/K_M can be limited by the product release (see the discussion on the Haldane relationship below).

Generalized kinetic model

Now we derive the reciprocal expression of k_{cat}/K_M for a generalized kinetic model even without deriving the rate equation. Let's say the generalized kinetic model contains the free enzyme (E), n bound forms of the enzyme (E_b 's), one substrate (S), and one product (P):

The kinetic model ends with an irreversible step, which leads to E + P. The reciprocal form of k_{cat}/K_M of this generalized kinetic model is expressed as the sum of the reciprocals of rate constants corresponding to each transition state:

$$\begin{aligned} \frac{1}{k_{cat}/K_M} &= \frac{1}{k_1} + \frac{k_2}{k_1 k_3} + \frac{k_2 k_4}{k_1 k_3 k_5} + \dots + \frac{k_2 k_4 \dots k_{2n}}{k_1 k_3 k_5 \dots k_{2n+1}} \\ &= \sum_{i=0}^n \frac{\prod_{j=0}^i k_{2j}}{\prod_{j=0}^i k_{2j+1}}, \text{ where } k_0 = 1 \end{aligned}$$

(10)

This generalized description of k_{cat}/K_M clearly demonstrates that, in any kinetic model, k_{cat}/K_M has a straightforward physical meaning, which can be easily shown on the reaction energy diagram. As stated

above, the highest transition state out of the $n+1$ transition states in the reaction energy diagram has the dominant influence on k_{cat}/K_M .

Steps that are considered in k_{cat}/K_M

It is commonly stated that the expression of k_{cat}/K_M includes all the elementary rate constants from the free enzyme (E + S) to the first irreversible step (4). It is quite simple to demonstrate this property using the reciprocal expression of k_{cat}/K_M . By converting the reversible chemical conversion step ($\text{ES} \rightleftharpoons \text{EP}$) in Model 1 to an irreversible one ($\text{ES} \rightarrow \text{EP}$), we can make Model 3:

in which we do not have k_4 , which is the rate constant for the reverse reaction of the chemical conversion step ($\text{EP} \rightarrow \text{ES}$). Eliminating k_4 in Model 2 is mathematically identical to setting k_4 to 0 in the expression of k_{cat}/K_M for Model 2 (Eq. 9). Therefore, the reciprocal form of k_{cat}/K_M for Model 3 is:

$$\frac{1}{k_{\text{cat}}/K_M} = \frac{1}{k_1} + \frac{k_2}{k_1 k_3} \quad (11)$$

As we stated above, k_{cat}/K_M in Eq. 11 includes the elementary rate constants from the free enzyme to the first irreversible step ($\text{EP} \rightarrow \text{ES}$) in Model 3. We can also demonstrate the property with the generalized model (Model 2). In the reciprocal form of k_{cat}/K_M for the generalized model (Eq. 10), all the rate constants for the reverse reactions (rate constants with even subscripts, such as k_2, k_4, k_6 , etc.) are in the

numerators of the terms. By setting any of the rate constants with an even subscript to 0, the first term that contains the rate constant and all the terms following disappear from the expression of k_{cat}/K_M .

Any steps after the first irreversible step does not affect k_{cat}/K_M because k_{cat}/K_M describes the kinetics from E + S, and any species after the first irreversible step cannot go back to E + S. We can explain this more intuitively using the reaction energy diagram. The heights of the transition states determine k_{cat}/K_M . An irreversible step is where the rate constant for the reverse reaction is 0. Therefore, an irreversible step has an infinite drop in the forward direction in the reaction energy diagram, which we indicate as a downward arrow (Figure 2). Due to this infinite drop, transition states after the first irreversible step are infinitely lower than those before the irreversible step and cannot make any contribution to k_{cat}/K_M .

Barriers versus Troughs

Reaction energy diagrams for enzyme-catalyzed reactions are composed of barriers (transition states) and troughs (bound forms, such as ES and EP). It is known that the depths of the troughs do not affect k_{cat}/K_M (4). We already demonstrated above that k_{cat}/K_M is determined by the heights of the transition states. When the depth of any trough varies without affecting the heights of the neighboring barriers (Figure 3A), k_{cat}/K_M stays the same.⁹ In other words, k_{cat}/K_M reports how effectively the enzyme stabilizes the transition states, regardless of its stabilization of the bound forms (or affinities to the substrate, the product, or any intermediates in between). One can verify this using the reciprocal expression of k_{cat}/K_M . When the energy level of ES is raised (Figure 3A), both k_2 and k_3 are increased to the same degree, which means that the ratio of k_2 to k_3 (k_2/k_3) remains unaffected. As one can verify in Eqs. 9 and 11, k_{cat}/K_M does not change when k_2/k_3 remains constant.

Now we consider the case that the height of only one of the barriers is increased. Such a case is common in kinetic studies on isotope effects (1). When a covalent bond breaks or forms during the chemical conversion step ($\text{ES} \rightleftharpoons \text{EP}$), the replacement of an atom with a heavier isotope selectively slows the chemical conversion step (Figure 3B). In that case, the height of the transition state is increased, and

k_{cat}/K_M may decrease. However, the degree of the change in k_{cat}/K_M depends on the degree of the influence of the chemical conversion step on k_{cat}/K_M . If the transition state of the chemical conversion step is the highest barrier, the isotope effect on k_{cat}/K_M is apparent (Figure 3B). If the transition state of the chemical conversion step is not the highest barrier, the isotope effect on k_{cat}/K_M is significantly suppressed or even undetectable because k_{cat}/K_M is determined mostly by a transition state that is not affected by the presence of the isotope.

Haldane Relationship

Many of the enzyme-catalyzed reactions are reversible, and enzymes can catalyze both forward and reverse reactions. Moreover, the physiological function of some enzymes, such as adenylate kinase and triose phosphate isomerase, is to maintain the equilibrium between the substrate and the product in cells. For reversible reactions catalyzed by an enzyme, the following relationship holds true:

$$\frac{(k_{\text{cat}}/K_M)_f}{(k_{\text{cat}}/K_M)_r} = K_{\text{eq}} \quad (12)$$

where $(k_{\text{cat}}/K_M)_f$ is k_{cat}/K_M for the forward reaction, $(k_{\text{cat}}/K_M)_r$ is k_{cat}/K_M for the reverse reaction, and K_{eq} is the equilibrium constant of the reaction. This relationship was originally discovered by Haldane and known to be the Haldane relationship (14). As long as only a catalytic amount of an enzyme is present, the presence of an enzyme does not affect K_{eq} . Eq. 12 shows that k_{cat}/K_M for the forward reaction and k_{cat}/K_M for the reverse reaction are mathematically tied together by the equilibrium constant. When one of the values is increased or decreased, the other also varies in the same direction proportionally. Therefore, the Haldane relationship is another constraint on the limit of k_{cat}/K_M . For example, if K_{eq} is significantly smaller than 1, k_{cat}/K_M for the forward reaction cannot reach to the diffusion limit. According to Eq. 12, when K_{eq} is significantly smaller than 1, $(k_{\text{cat}}/K_M)_r$ is much greater than $(k_{\text{cat}}/K_M)_f$. If $(k_{\text{cat}}/K_M)_f$ is diffusion-

controlled, $(k_{\text{cat}}/K_M)_r$ should be greater than the diffusion limit, which is physically impossible. Likewise, if K_{eq} is significantly larger than 1, k_{cat}/K_M for the reverse reaction cannot reach the diffusion limit.

The Haldane relationship can be understood more intuitively by using the reaction energy diagram. Let's construct the kinetic model for a reversible reaction by adding the rate constant for the product association (k_6) to Model 1:

To experimentally determine $(k_{\text{cat}}/K_M)_f$ for a reversible reaction, one keeps the product concentration close to 0 so that the reverse reaction is negligible. This experimental condition is mathematically identical to setting k_6 to 0, which makes the kinetic model same as Model 1 and the reciprocal form of $(k_{\text{cat}}/K_M)_f$ same as Eq. 9:

$$\frac{1}{(k_{\text{cat}}/K_M)_f} = \frac{1}{k_1} + \frac{k_2}{k_1 k_3} + \frac{k_2 k_4}{k_1 k_3 k_5} \quad (13)$$

Likewise, we determine the expression for $(k_{\text{cat}}/K_M)_r$ by setting k_1 to 0:

$$\frac{1}{(k_{\text{cat}}/K_M)_r} = \frac{1}{k_6} + \frac{k_5}{k_4 k_6} + \frac{k_3 k_5}{k_2 k_4 k_6} \quad (14)$$

Now let's assume that one transition state is significantly higher than others. While Haldane relationship does not require this assumption, we can use this assumption to simplify the demonstration. It

is obvious that the highest transition state for the forward reaction is also the highest transition state for the reverse reaction. The same step has the dominance influence on both $(k_{\text{cat}}/K_M)_f$ and $(k_{\text{cat}}/K_M)_r$. In other words, the same step is rate-limiting for both $(k_{\text{cat}}/K_M)_f$ and $(k_{\text{cat}}/K_M)_r$. In case of the reaction energy diagram in Figure 4, the chemical conversion step ($\text{ES} \rightleftharpoons \text{EP}$) has the highest transition state, and $(k_{\text{cat}}/K_M)_f$ and $(k_{\text{cat}}/K_M)_r$ can be approximated as:

$$(k_{\text{cat}}/K_M)^f = \frac{k_1 k_3}{k_2} \quad (15)$$

$$(k_{\text{cat}}/K_M)^r = \frac{k_4 k_6}{k_5} \quad (16)$$

Now using the above two equations, one can show that the ratio of $(k_{\text{cat}}/K_M)_f$ to $(k_{\text{cat}}/K_M)_r$ is identical to K_{eq} , the equilibrium constant of the reaction:

$$\frac{(k_{\text{cat}}/K_M)^f}{(k_{\text{cat}}/K_M)^r} = \frac{k_1 k_3 k_5}{k_2 k_4 k_6} = K_{\text{eq}} \quad (17)$$

One can easily verify that this relationship holds when the highest transition state is the substrate association step ($\text{E} + \text{S} \rightleftharpoons \text{ES}$) or the product release step ($\text{EP} \rightleftharpoons \text{E} + \text{P}$). Whatever transition state is chosen, $(k_{\text{cat}}/K_M)_f$ includes all the elementary rate constants from $\text{E} + \text{S}$ to the transition state, and $(k_{\text{cat}}/K_M)_r$ includes all the elementary rate constants from $\text{E} + \text{P}$ to the same transition state. As shown in Eq. 17, the ratio of $(k_{\text{cat}}/K_M)_f$ to $(k_{\text{cat}}/K_M)_r$ contains the product of all the forward rate constants in the denominator and the product of all the reverse rate constants in the numerator, which is equivalent to K_{eq} .

One can also prove the Haldane relationship by simply calculating the ratio of $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$ to $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$ in Eqs. 14 and 15 without assuming any single transition state is significantly higher than other transition states. Because the forward reaction and the reverse reaction go through the same transition states simply in the reverse order, the relative contribution of each transition state is identical to $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$ and $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$. Because $k_{\text{cat}}/K_{\text{M}}$ is expressed as the sum of the kinetic terms corresponding to the transition states (Eq. 9), reversing the order of the kinetic terms in the expression does not change the sum. The only difference between $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$ and $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$ is the starting point (or the reference level for the transition states), E + S and E + P, respectively, and this difference makes the ratio of the two parameters K_{eq} .

As we stated already, the same step is rate-limiting for both $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$ to $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$. If the substrate-association step is rate-limiting for $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$, the same step, which is the product-release step in the reverse reaction, is also rate-limiting for $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$. From this principle, one can deduce intuitively that it is not possible for an enzyme to have $k_{\text{cat}}/K_{\text{M}}$ at the diffusion limit for both forward and reverse reactions, which we have proved above using the Haldane relationship. If $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$ is at the diffusion limit, the substrate-association step is rate-limiting for $(k_{\text{cat}}/K_{\text{M}})_{\text{f}}$, which means that the product-release step should be rate-limiting for $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$. Therefore, the substrate-association step cannot be rate-limiting for $(k_{\text{cat}}/K_{\text{M}})_{\text{r}}$.¹⁰

Conclusion

$k_{\text{cat}}/K_{\text{M}}$ is frequently misunderstood as a composite kinetic parameter derived from k_{cat} and K_{M} . Even many biochemistry textbooks wrongfully state that $k_{\text{cat}}/K_{\text{M}}$ is a way to consider both catalytic power (k_{cat}) and binding affinity (K_{M}) of the enzyme for its substrate. Different from k_{cat} and K_{M} , $k_{\text{cat}}/K_{\text{M}}$ has a clear and straightforward meaning that can be visualized in the reaction energy diagram; $k_{\text{cat}}/K_{\text{M}}$ corresponds to the height of the highest transition state that the enzyme needs to cross along the reaction pathway of the enzyme-catalyzed reaction. Even in a minimal kinetic model as Model 1, $k_{\text{cat}}/K_{\text{M}}$ can be limited by the substrate association, the chemical conversion, or even the product release. Thus, using $k_{\text{cat}}/K_{\text{M}}$ to consider both catalytic power and binding affinity is misleading. For example, it is quite common that $k_{\text{cat}}/K_{\text{M}}$ is

limited by the substrate association, i.e. $k_{\text{cat}}/K_M \sim k_1$, and k_1 has nothing to do with the catalytic power or the binding affinity of the enzyme. The proper understanding of k_{cat}/K_M using the reaction energy diagram is extremely helpful to analyze the effects of mutations in the enzyme, structural variations in the substrate, modification of the experimental condition, and so forth. When any of these changes affects the energy of the highest transition state, we observe a change in k_{cat}/K_M . And, the extent of the change we observe in k_{cat}/K_M provides valuable information on the reaction energetics and catalytic mechanism of the enzyme. Moreover, the primary mechanism for the enzyme to catalyze a reaction is to lower the energy of the transition states, and k_{cat}/K_M is a direct way to observe the outcome of this stabilization (15, 16). This is why enzymologists consider k_{cat}/K_M along with k_{cat} as a fundamental kinetic parameter in enzyme kinetics and consider K_M as simply the ratio of the two fundamental kinetic parameters (1, 5, 6). Finally, I want to point out that the common practice to determine k_{cat} and K_M first by fitting the Michaelis–Menten equation to the experimental data and calculate k_{cat}/K_M later using the two fitted parameters is not desirable. This practice produces a k_{cat}/K_M value with an unnecessarily exaggerated fitting error due to the error propagation. Considering that k_{cat}/K_M is such a fundamental kinetic parameter, one should avoid the error propagation by determining k_{cat} and k_{cat}/K_M directly from the fitting of the Michaelis–Menten equation with the following fitting function: $f = a^*x/(a/b + x)$, where $f = v/E_t$, $x = [S]$, $a = k_{\text{cat}}$ and $b = k_{\text{cat}}/K_M$. Then, one can calculate K_M from k_{cat} and k_{cat}/K_M , only if it is necessary.

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Table 1. Influence of individual kinetic terms on k_{cat}/K_M in Eq. 9. k_1 is fixed at $10^6 \text{ M}^{-1}\text{s}^{-1}$, and k_1k_3/k_2 and $k_1k_3k_5/k_2k_4$ are varied from $10^5 \text{ M}^{-1}\text{s}^{-1}$ to $10^7 \text{ M}^{-1}\text{s}^{-1}$. The least terms, which have the dominant influence on k_{cat}/K_M , are shown in bold.

k_1 ($\times 10^6 \text{ M}^{-1}\text{s}^{-1}$)	k_1k_3/k_2 ($\times 10^6 \text{ M}^{-1}\text{s}^{-1}$)	$k_1k_3k_5/k_2k_4$ ($\times 10^6 \text{ M}^{-1}\text{s}^{-1}$)	k_{cat}/K_M ($\times 10^6 \text{ M}^{-1}\text{s}^{-1}$)
1	0.1	0.1	0.048
1	0.1	1	0.083
1	0.1	10	0.09
1	1	0.1	0.083
1	1	1	0.33
1	1	10	0.48
1	10	0.1	0.09
1	10	1	0.48
1	10	10	0.83

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Figure Legends

Figure 1. Plot of v/E_t versus $[S]$ according to the Michaelis–Menten equation. At saturating concentrations of $[S]$, v/E_t approaches at k_{cat} . At low concentrations of $[S]$, v/E_t increases linearly with the slope of k_{cat}/K_M as $[S]$ increases.

Figure 2. Normative reaction energy diagrams for the kinetic model shown in Model 1. The heights of the transition states corresponding to k_1 , k_1k_3/k_2 , and $k_1k_3k_5/k_2k_4$ relative to $E + S$ are indicated by teal arrows. **(A)** The transition state for the substrate-association step ($E + S \rightarrow ES$) is the highest. **(B)** The transition state for the chemical-conversion step ($ES \rightarrow EP$) is the highest. **(C)** The transition state for the product-release step ($EP \rightarrow E + P$) is the highest.

Figure 3. Effects of the change in the energy levels of a trough and a barrier on k_{cat}/K_M . The heights of the transition states corresponding to k_1 , k_1k_3/k_2 , and $k_1k_3k_5/k_2k_4$ relative to $E + S$ are indicated by teal arrows. **(A)** The energy level of the ES complex is increased. **(B)** The energy level of the transitions state for the chemical conversion step ($ES \rightarrow EP$) is increased.

Figure 4. k_{cat}/K_M of the forward reaction and the reverse reaction of a reversible reaction. The transitions state for the chemical conversion step ($ES \rightarrow EP$) is the highest, and the height of the transition state relative to $E + S$ and $E + P$ are indicated by teal arrows corresponding to k_{cat}/K_M for the forward reaction and the reverse reaction, respectively.

A

B

C

A

B