Enzyme kinetics based on free-energy profiles

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A comprehensive theory for relating free-energy profiles to kinetic equations of enzyme reactions has been developed. It enables expression of the overall rate and the concentrations of reaction intermediates in terms of the heights of peaks in free-energy profiles for various reactions. The reactions include consecutive reactions with intermittent irreversible steps, those with dead-end side reactions and completely reversible reactions. The usefulness of the theory is shown by analysis of a single-substrate reaction, a reaction with a covalent intermediate, and

product inhibition in a two-substrate reaction, in which kinetic parameters such as $V_{\max}$ and K_m are related to the peak heights in free-energy profiles. The paper refers also to the concept of rate-determining step (RDS) and shows the utility of a related concept rate-determining zone (RDZ) which indicates the reaction steps between the main intermediate and the RDS. The concept of RDZ is useful for resolving confusion related to the concept of RDS. It should be emphasized that the present approach is applicable only to linear reactions.

INTRODUCTION

Free-energy profiles have been used to discuss the mechanisms of both chemical and enzymic reactions. They simplify complicated ideas and allow a basic consideration of the reaction mechanisms. Enzyme mechanisms such as transition-state stabilization, strain mechanism and induced-fit mechanism can be readily explained by using free-energy profiles [1,2]. In recent research based on protein engineering, the effects of amino acid replacement are also often discussed in terms of free energy or related concepts [3,4]. However, no systematic method for relating free-energy profiles to ordinary kinetics has been developed, although basic relationships between kinetic expressions and free-energy profiles have been elucidated [5–7]. The purpose of the present study was to develop a simple procedure for obtaining such relationships in the hope of providing new insights into, and deeper understanding of, enzyme mechanisms. Despite the apparent mathematical complexity of the derivation of the theory, the result is so simple that, in our laboratory, enzyme mechanisms are now normally discussed in terms of free-energy profiles rather than kinetic equations.

THEORY

Kinetic equations related to free-energy profiles

Linear reactions with an irreversible final step

As shown elsewhere [7], when we consider an enzyme reaction:

and define the overall reaction rate by:

$$v = d[P]/dt = k_{+n}[E_n] \quad (2)$$

the steady-state conditions:

$$d[E_i]/dt = 0 \quad (i = 1 \text{ to } n) \quad (3)$$

give:

$$k_{+i}[E_i] - k_{-i}[E_{i+1}] = v \quad (i = 1 \text{ to } n-1) \quad (4)$$

Here, the term $k_s[S]$ will be replaced with k_{+1} for simplicity of expression, until after the effect of the substrate concentration

has been considered. Eqn. (4) indicates that the net flow at each step is equal to the overall rate. Eqns. (2) and (4) arranged in the matrix:

$$\begin{pmatrix} k_{+1} & -k_{-1} & & & & \\ & k_{+2} & -k_{-2} & & & \\ & & k_{+3} & -k_{-3} & & \\ & & & - & - & \\ & & & & - & - \\ & & & & & k_{+(n-1)} & -k_{-(n-1)} \\ & & & & & & k_{+n} \end{pmatrix} \begin{pmatrix} E_1 \\ E_2 \\ E_3 \\ - \\ - \\ E_{n-1} \\ E_n \end{pmatrix} = \begin{pmatrix} v \\ v \\ v \\ - \\ - \\ v \\ v \end{pmatrix} \quad (5)$$

give eqn. (6) as the solution:

$$[E_i] = \left(\frac{1}{k_{+i}} + \frac{k_{-i}}{k_{+i}k_{+(i+1)}} + \frac{k_{-i}k_{-(i+1)}}{k_{+i}k_{+(i+1)}k_{+(i+2)}} + \dots + \frac{k_{-i}k_{-(i+1)} \dots k_{-(n-1)}}{k_{+i}k_{+(i+1)} \dots k_{+(n-1)}k_{+n}} \right) v \quad (6)$$

Summing the equations represented by eqn. (6) for $i = 1$ to n leads to:

$$v = E_0 \sum_{i=1}^n \left(\frac{1}{k_{+i}} + \frac{k_{-i}}{k_{+i}k_{+(i+1)}} + \dots + \frac{k_{-i}k_{-(i+1)} \dots k_{-(n-1)}}{k_{+i}k_{+(i+1)} \dots k_{+(n-1)}k_{+n}} \right) \quad (7)$$

where E_0 denotes the total enzyme concentration, $\sum E_i$. Substitution of v in eqn. (6) by eqn. (7) gives $[E_i]$ expressed by k_i s and E_0 .

Each term in eqns. (6) and (7), when expressed in a logarithmic form, is proportional to the height of a peak measured from the level of E_i in free-energy profiles as illustrated in Figure 1. Because the height of a peak (ΔG) in a free-energy profile based on the transition-state theory is proportional to

Figure 1 Values of terms in eqn. (6) depicted on the free-energy profile

The lengths of the arrows represent the terms in eqn. (6) in a logarithmic form.

Figure 2 Values of terms in eqn. (6) depicted on the free-energy profile intermitted by irreversible steps

$RT \ln (1/k_i)(h/kT)$, where R is the gas constant per mole, T is temperature h is the Planck constant and k is the Boltzmann constant, then the terms in eqns. (6) and (7) in the logarithmic form are represented by the summation (for the forward reactions) and the subtraction (for the reverse reactions) of ΔG values. From this view, eqn. (6) means that the intermediates are deposited in proportion to the heights of the barriers that they must overcome to reach the final product, and eqn. (7) means that the overall rate is inversely proportional to the sum of the peak heights measured from the level of all the intermediates. Equations essentially equivalent to (6) and (7) have been derived by different methods [5,6,8] and their correlations with the free-energy profile also pointed out.

Sequential reactions with irreversible steps

For reactions involving more than one irreversible step, the terms in eqns. (6) and (7) can be related to the peak heights measured independently in the parts separated by the irreversible steps as shown in Figure 2. In other words, eqns. (6) and (7) do not involve terms spanning an irreversible step, as they become zero involving a rate constant of zero for the irreversible step.

Figure 3 Free-energy profile with a quasiequilibrium step

(a) The condition for quasiequilibrium, namely eqn. (8), holds when the dashed arrow is shorter than the sum of the solid arrows. (b) Eqn. (8) does not hold in this case. (c) For the profile in (a), the dashed arrows starting from all preceding troughs are shorter than the sum of the solid arrows starting from the same troughs.

Sequential reactions with quasiequilibrium steps

Quasiequilibrium between two intermediates, E_i and E_{i+1} , can be defined as:

$$\frac{E_i}{E_{i+1}} \approx \frac{k_{-i}}{k_{+i}} \quad (8)$$

and eqn. (6) gives:

$$\frac{E_i}{E_{i+1}} = \frac{\frac{1}{k_{+i}} + \frac{k_{-i}}{k_{+i}} \left[\frac{1}{k_{+(i+1)}} + \frac{k_{-(i+1)}}{k_{+(i+1)}k_{+(i+2)}} + \dots + \frac{k_{-(i+1)} \dots k_{-(n-1)}}{k_{+(i+1)} \dots k_{+n}} \right]}{\frac{1}{k_{+(i+1)}} + \frac{k_{-(i+1)}}{k_{+(i+1)}k_{+(i+2)}} + \dots + \frac{k_{-(i+1)} \dots k_{-(n-1)}}{k_{+(i+1)} \dots k_{+n}}} \quad (9)$$

Eqn. (8) holds when the first term in the numerator of eqn. (9) is much smaller than the rest of the terms, in other words, when the arrow representing the first term is much shorter than the sum of the lengths of the arrows for all succeeding steps as shown in Figure 3(a). The requirement may be regarded as being usually fulfilled when the arrow for the i th peak is somewhat shorter than the highest of the succeeding peaks, because the comparison should be made on a normal scale, and a small difference in a logarithmic scale represents a large difference in the normal scale. Eqn. (8) does not hold for the case of Figure 3(b) which differs

from Figure 3(a) in the value of k_{-i} . It is apparent that the value of k_{-i} , not the value of k_{+i} , represented by the height of the i th peak measured from behind, is the primary factor deciding the validity of the quasiequilibrium approximation as pointed out for simpler cases [9]. In the case of Figure 3(a), all the terms ending with $1/k_i$ in eqn. (6) represented by dotted arrows in Figure 3(c) should also be much smaller than the sum of the succeeding terms. Then, for reactions involving quasiequilibrium steps, eqns. (6) and (7) can be represented by the sets of arrows except those pointing to the i th peak.

Sequential reactions with dead-end side reactions

For the reactions written as:

the steady-state equation can be derived by using the fact that eqn. (6), which gives the concentrations of the intermediates in linear reactions, holds also for the intermediates on the main route of the above reaction, since eqn. (4), which indicates the equality of the net flows, can be applied to the steps in the main route of reaction (10). Then eqn. (6) combined with eqn. (11), which represents the null net flow for the side reactions, gives eqn. (12).

$$k'_{-i}E_i - k'_{+i}E'_i = 0 \quad (11)$$

$$[E'_i] = \frac{k'_{-i}}{k'_{+i}} \left[\frac{1}{k_{+i}} + \frac{k_{-i}}{k_{+i}k_{+(i+1)}} + \dots + \frac{k_{-i}k_{-(i+1)} \dots k_{-(n-1)}}{k_{+i}k_{+(i+1)} \dots k_{+(n-1)}k_n} \right] v \quad (12)$$

Eqn. (12) indicates that, as illustrated in Figure 4, the concentration of the intermediates in the side reactions, E'_i , can also be represented by the set of arrows drawn from the level of E'_i to the peaks that must be overcome to reach the final state, although no arrow should be drawn to the peaks in the side reactions, as there is no term ending with $1/k'_i$ in eqn. (12). Summing of the concentrations of all the intermediates expressed

Figure 4 Free-energy profile for dead-end side reactions

The concentration of the side intermediate is inversely proportional to the sum of the arrow lengths.

by both eqns. (6) and (12) leads to an expression of the overall rate that is similar to eqn. (7) but involves the terms for the side intermediates.

Reversible reactions

For reactions with a reversible final step written as:

steady-state conditions lead to eqns. (14) and (15), where $k_s S$ and $k_p P$ are replaced by k_{+1} and k_{-n} respectively:

$$v = k_{+i}E_i - k_{-i}E_{i+1} \quad (i = 1 \text{ to } n-1) \quad (14)$$

$$v = k_{+n}E_n - k_{-n}E_1 \quad (15)$$

Eqns. (14) and (15) arranged in a matrix:

$$\begin{pmatrix} k_{+1} & -k_{-1} & & & & \\ & k_{+2} & -k_{-2} & & & \\ & & k_{+3} & -k_{-3} & & \\ & & & - & - & \\ & & & & - & - \\ & & & & & k_{+(n-1)} & -k_{-(n-1)} \\ -k_{-n} & & & & & & k_{+n} \end{pmatrix} \begin{pmatrix} E_1 \\ E_2 \\ E_3 \\ \vdots \\ E_{n-1} \\ E_n \end{pmatrix} = \begin{pmatrix} v \\ v \\ v \\ \vdots \\ v \\ v \end{pmatrix} \quad (16)$$

give eqn. (17) where K_i denotes k_{-i}/k_{+i} :

$$E_i = \left[\frac{1/k_{+i} + K_i/k_{+(i+1)} + K_iK_{i+1}/k_{+(i+2)} + \dots + K_iK_{i+1} \dots K_{n-1}/k_{+n}}{1 - K_1K_2K_3 \dots K_n} \right] v \quad (17)$$

The numerator of eqn. (17) is represented by the arrows in Figure 5, in which two free-energy profiles are combined in tandem by equalizing the final free-energy level of the first one with the initial level of the second one. In the combined profile, arrows are also drawn to the peaks in the second profile from the level of the troughs in the first profile. The arrows start from the i th peak and end at the $(i-1)$ th peak of the second profile. Physical meanings of the tandem profile have not been clarified yet and it will be used just as a tool for describing the reversible sequential reactions in the present theory.

Figure 5 Tandem free-energy profile depicting the terms in eqn. (17)

Summation of eqn. (17) for all i values gives:

$$v = \frac{E_0(1 - K_1 K_2 K_3 \dots K_n)}{\sum_{i=1}^n A_i} \quad (18)$$

where A_i denotes the numerator of eqn. (17). Eqn. (18) changes its sign depending on the value of $K_1 K_2 K_3 \dots K_n$ which is the chemical equilibrium constant multiplied by $[P]/[S]$ and is represented by the free-energy difference between the first and final states of a profile. The negative v value means the reaction rate in the reverse direction. The principles in the above three sections, such as the separate treatment of intermitted profiles, shown to be applicable to reactions with an irreversible final step, apply also to reactions with a reversible final step exhibited by tandem free-energy profiles.

Approximations in the interpretation of free-energy profiles

Approximation of the overall rate by the maximum free-energy difference

If one of the peak heights measured by the method in Figure 1 is much larger than the sum of all the others, in other words, when one of the terms in eqn. (7) predominates over the others, eqn. (7) can be approximated by:

$$v = E_0 \left/ \left[\frac{k_{-p} k_{-(p+1)} \dots k_{-(q-1)}}{k_{+p} k_{+(p+1)} \dots k_{+(q-1)} k_{+q}} \right] \right. \quad (19)$$

where k_p and k_q are the rate constants defined by the trough and the peak giving the maximum ΔG .

Approximation by two major free-energy differences

Figure 6 shows the free-energy profiles with two major peaks of the same height. The overall rates for Figures 6(a) and 6(b) are one-half of that for a profile with only one of the two peaks, whereas that for Figure 6(c) is one-third of the single-peak profile, because the denominator of eqn. (7) giving the overall rate is the sum of the length of the arrows. Situations represented by the former two cases occur when enzyme reactions take place at a substrate concentration equal to K_m or at an inhibitor concentration equal to K_i . The two predominant peaks can also represent the presence of two predominant intermediates of an equal amount, as can be deduced from eqn. (6). The two approximations apply to all reactions described hitherto including wholly reversible ones which should be represented by tandem free-energy profiles.

Figure 6 Free-energy profile with two major free-energy differences

The contributions of the dotted arrows are ignored.

Rate-determining step (RDS), main intermediate and rate-determining zone (RDZ).

The step represented by k_q in eqn. (19) is the RDS in the sense that a change in this rate constant only results in a significant change in the overall rate, as long as the overall rate is approximated by the equation and the change in the rate constants is assumed to result from the change in the free energy of the transition states. A change in the transition state results in a simultaneous change in the forward and reverse rate constants in the same ratio. The change in rate constants in this manner has no effect on the values of eqn. (19), being cancelled out in the denominator and numerator of the equation except for the case of k_q . The main intermediate is represented by E_p as can be explained by using eqn. (6). When the mode of change in the rate constants is not restricted as above, all the rate constants in eqn. (19) affect the overall rate equally. Therefore this part of the sequential reaction, namely the steps between the main intermediate and the RDS, will be called the RDZ.

Enzyme kinetics based on free-energy profiles

Free-energy profiles at varied substrate and inhibitor concentrations

In the free-energy profiles used in the preceding sections, the height of the first peak was assumed to be proportional to $RT \ln(1/k_s S)(h/kT)$. This requirement is fulfilled when the free energy of the substrate at a concentration of S is defined by eqn. (20), where G_s° denotes the free energy of the substrate in the standard state, and the free energy of the E+S state given by eqn. (21), namely the sum of the standard free energy of the enzyme and the real free energy of the substrate, is plotted. The physical meaning of this type of free-energy profile will be discussed below.

$$G_s = G_s^\circ + RT \ln S \quad (20)$$

$$G_{E+S} = G_E^\circ + G_s^\circ + RT \ln S \quad (21)$$

The same rule can be applied to the plot of the states involving inhibitors or other additives. Another point to be considered in the plotting of free-energy profiles is the overall stoichiometry of the reaction co-ordinate [10]. For example, in reactions with an added inhibitor, all the states, except those involving bound inhibitor such as EI and (E+I)*, should involve free inhibitor, and therefore the free energies of all the states, including transition states, should involve that of the inhibitor as exemplified in eqn. (22).

$$G_{ES+I} = G_{ES}^\circ + G_I^\circ + RT \ln I \quad (22)$$

Therefore the vertical levels of all the states, except EI and (E+I)*, go up by $G_I^\circ + RT \ln I$ when an inhibitor is added. The principle of the overall stoichiometry requires also that even for reactions without added product, the final state of the profile should be an E+P state, where P denotes the presence of 1 mol of product at an infinitely low concentration which gives an infinitely deep trough because of the term $RT \ln 0$.

Single-substrate reaction

For reaction (23), the steady-state rate can be expressed by eqn. (24) according to eqn. (6).

$$v = E_0 \left/ \left(\frac{1}{k_{+1} S} + \frac{k_{-1}}{k_{+1} S k_{+2}} + \frac{1}{k_{+2}} \right) \right. \quad (24)$$

Figure 7 Free-energy profiles for the Michaelis-Menten mechanism

The dotted curve depicts the profile for the standard state of S.

Figure 7 shows the free-energy profiles of a reaction in which $k_{+2} \ll k_{-1}$, i.e. a reaction that obeys Michaelis-Menten kinetics. Curves A, B and C show the profiles at three different substrate concentrations. The free-energy differences designated by arrows a, b and c correspond to the three terms in eqn. (24). Curve A is the profile for a low substrate concentration. The $\Delta G_{\max.}$ that approximates the overall rate is shown by arrow b in this case. When S increases, the free energy of $E + S$ goes up and arrow b becomes shorter, as shown by the dotted arrows. At a value of S (curve B), its length becomes equal to that of arrow c. Further increase in S (curve C) does not change the $\Delta G_{\max.}$, which is now represented by arrow c. This corresponds to the levelling off of the reaction rate at high substrate concentrations, and the maximum velocity $V_{\max.}$ or the corresponding rate constant $k_{\text{cat.}}$ is represented by arrow c. The K_m value is given by the substrate concentration that equalizes arrow b with arrow c. At this

concentration of S , the overall rate, v , becomes half of that represented by arrow c and the concentrations of E and ES become equal for the reasons already noted. The actual value of S in the form of $RT \ln S$ is depicted by arrow d, and is the difference between curve B and the dotted curve representing the standard state. The downward facing arrowhead of arrow d indicates a value of S smaller than unity. The value of $k_{\text{cat.}}/K_m$ is depicted by arrow e, because the rate at an infinitely dilute S is $k_{\text{cat.}} S/K_m$ and should be divided by S to give $k_{\text{cat.}}/K_m$.

Similar analysis of the Briggs-Haldane mechanism by using profiles in which the first peak is higher than the second ($k_{-1} \ll k_{+2}$) will clarify the characteristics of the mechanism. Drawing the profile as a reversible reaction will also demonstrate that there is no reaction for which the Briggs-Haldane mechanism is valid in both directions [11].

Reactions with a covalent intermediate

Figure 8 shows two types of free-energy profile for reaction (25) which can be represented by the acyl-enzyme mechanisms for proteinases.

$$v = E_0 / \left(\frac{1}{k_{+1}S} + \frac{k_{-1}}{k_{+1}Sk_{+2}} + \frac{1}{k_{+2}} + \frac{1}{k_{+3}} \right) \quad (26)$$

Eqn. (26) can be derived by merely adding the term representing the final peak ($1/k_{+3}$) to eqn. (24). Figure 8(a) shows the case where $k_{+2} \gg k_{+3}$ and Figure 8(b) shows the case where $k_{+2} \ll k_{+3}$. The profiles are drawn for situations in which the substrate concentrations are very low (curve A), equal to K_m (curve B) and very high (curve C). In both Figures 8(a) and 8(b), the overall rate at low substrate concentration is represented by arrow b. $V_{\max.}$ is represented by the longest arrow in curve C, which is arrow d in Figure 8(a) and arrow c in Figure 8(b). At $S = K_m$, the length of arrow b becomes the same as that of arrow d in Figure 8(a) and as arrow c in Figure 8(b). The contribution of arrow a which is always shorter than b is ignored. The value of $k_{\text{cat.}}/K_m$ is represented by arrow b cut at the level of the standard state of S . Therefore an important relationship in this type of reaction, i.e. that $k_{\text{cat.}}/K_m$ is equal to k_{+2}/K_s and not affected by k_{+3} even in the case of $k_{\text{cat.}} = k_{+3}$, can be readily explained by the profiles.

Product inhibition in a two-substrate reaction

The compulsory-ordered reactions expressed in reaction (27) will be considered as an example of a two-substrate reaction.

Figure 9 shows the free-energy profiles for the reaction at a saturating concentration of B in the presence of Q and the absence of P . In Figure 9, two profiles are connected in tandem to express the reaction with the reversible final step. Each profile involves two apparently irreversible steps, one between arrow b and c due to the infinitely high concentration of B , the other, between arrow d and e due to the infinitely low concentration of P . The principle of separate treatment of intermitted profiles can be applied to these steps and the rate at moderate concentrations of A can be given without approximation as

$$v = E_0 / (a + c + d + d' + e + f) \quad (28)$$

where the bold letters denote the terms represented by the arrows designated by the same letters in Figure 9. Here, the contribution

Figure 8 Free-energy profiles for the acyl-enzyme mechanism

Figure 9 Free-energy profiles for a compulsory-ordered reaction in the presence of product Q

of arrows b and g representing the terms involving $1/k_{+2}B$ can be ignored. Furthermore, the arrows that follow the first irreversible step in the second profile do not contribute to the equation. The term $K_1K_2\dots K_n$ in eqn. (18) is also zero because of the involvement of the irreversible steps. At a high concentration of A, the contribution of the arrows a and f can be ignored and the value of eqn. (28), the apparent $V_{\max}$, is given by:

$$V_{\max, \text{app.}} = E_0/(c + d + d' + e) \quad (29)$$

The apparent K_m^A is the concentration of A that equalizes the sum of the arrows a and f with that of the arrows c, d, d' and e, and so it can be expressed as:

$$K_{m, \text{app.}}^A = \frac{1}{\frac{k_{+1}}{c + d + d' + e} + \frac{k_{-5}Q}{k_{+5}k_{+1}}} \quad (30)$$

The concentration of Q determines the level of the final state $E + P + Q$ and affects only the length of arrow f; therefore it does not affect the apparent $V_{\max}$, but affects the apparent K_m^A , i.e. the concentration of A equalizing $a + f$ with $c + d + d' + e$. This competitive mode in the inhibition of A by Q is a characteristic of the compulsory-ordered mechanism.

DISCUSSION

The two principles expressed by eqns. (6) and (7) for irreversible reactions and eqns. (17) and (18) for reversible reactions, relating the amount of the intermediates and the overall rate to peak heights of free-energy profiles, have been shown to be applicable to various types of reaction. The two approximations, that $\Delta G_{\max}$ represents the overall rate and two predominant ΔG values halve the rate, greatly simplify the explanation of free-energy profiles. A fact to be noted here is that eqns. (7) and (18) and the related arguments apply only to enzyme reactions or reactions in which the rate expressed in terms of total reactant concentration is the primary concern. For reactions for which the rate is expressed as a function of the concentration of the first reactant, i.e. typical chemical reactions, the maximum peak height measured from the level of the first reactant should be used in similar approximations [7]. Eqns. (6) and (17) exhibiting the amounts of intermediates hold for both kinds of reaction as long as the steady-state condition is fulfilled.

The matrices used in the present theory involve only single

terms such as k_i at limited positions and are simpler than those used by King and Altman [12] which are direct expressions of steady-state conditions and involve summed terms such as $k_{-(i-1)} + k_{+i}$. The matrix used here, which may be called a 'net-flow matrix' representing equality of net flows for all the steps of sequential reactions, is closely related to the concept of the net rate constant proposed by Cleland [8]. He defined the net rate constant, k'_i , as the rate constant giving the net flow of a step in the form of $v = k'_i E_i$ and utilized it to derive rate equations. Eqn. (6), the solution of the matrix, merely represents the relationship defined by Cleland, and so the reciprocal of the right-hand side of this equation, excluding v , is equal to the net rate constant, k'_i . Eqn. (17) also gives the net rate constant for reversible reactions.

As shown above, free-energy profiles are efficient tools for deriving kinetic expressions for various reactions; however, they can be applied only to linear reactions. The diagrammatic method developed for the same purpose by King and Altman [12] is superior in its generality. However, the present method is more intimately related to reaction mechanisms and gives kinetic expressions with clear physical meanings. A comparison of the two methods shows that, in the present method, the amount of each intermediate is expressed by the height of the barrier to be overcome, in other words, by the tendency to flow out towards the end of the reaction, whereas, in the King-Altman method, it is expressed by the tendency to flow in towards the intermediate via all possible routes.

In principle, free-energy profiles based on transition-state theory should depict the free energies of the reaction components and the transition states at the standard states, because the transition-state theory postulates an equilibrium between the reactants and the transition states, whereas the equilibrium constants are related to their standard free energies [10]. In this respect, the present free-energy profiles are anomalous. This anomaly, however, can be circumvented by defining the real concentrations of the substrates and inhibitors as new standard states [6]. However, re-definition of the standard states was not carried out in the present theory, because if done rigorously, it would introduce extra complexity into the theory accompanying the alteration of the units expressing both concentrations and rate constants. Another, more practical, explanation of the present profiles is to consider them as depicting 'apparent' standard free energies of enzyme-related species, because the free energies of the enzyme-related species including the transition states are assumed to be in standard states. Apparent standard free energies give apparent equilibrium constants which, for example, can be expressed as $[(E + S)^*]/[E]$ and $[ES]/[E]$ under defined conditions. Following this line of explanation, the free-energy difference between the first ($E + S$) and final ($E + P$) state can be explained as representing the apparent equilibrium constant between the free enzyme in the substrate and that in the product. The value is actually the free-energy difference between the substrate and the product at the defined concentrations.

The value of the present theory has been demonstrated here by some examples. Similar analysis of other reactions will provide new perspectives of the reactions. Various types of inhibition, such as competitive inhibition, non-competitive inhibition under rapid-equilibrium conditions, uncompetitive inhibition and inhibition by non-productive binding of substrates, can be analysed by the present method using the principles for branched reactions. However, non-competitive inhibition under steady-state conditions, which constitutes a two-route reaction, cannot be analysed by the present method. Multisubstrate linear reactions such as those that obey the Ping-Pong and Theorell-Chance mechanisms can also be analysed by the present method. The combined use of free-energy profiles and expressions in the

present style will clarify the physical meaning of kinetic parameters, which is often difficult using normal kinetics. At the least, the complex expressions for kinetic parameters such as $V_{\max}$ and K_m under various conditions can be derived from inspection of free-energy profiles.

Free-energy profiles have been determined only for a limited number of enzymes [3,13]. However, the present theory does not necessarily require complete free-energy profiles. Provisional free-energy profiles drawn using only limited kinetic data can be helpful for looking at different aspects of enzyme reactions. For example, in our laboratory, it led to the discovery of high-efficiency transpeptidation catalysed by a thiol proteinase clostripain [14]. The value of the present theory lies in the ability of free-energy profiles to describe complex situations. Theoretical approaches using quantum mechanics and molecular dynamics can be promising for the understanding of primary processes of enzyme reactions. However, enzyme reactions are principally multistep processes, and there are many problems in this research area that can be tackled by viewing them in terms of free energy, as shown by Jencks [1]. The study of complex biological processes such as muscle contraction and membrane transport also requires simple tools for elucidating complicated phenomena.

The RDS is an important concept in the discussion of reaction mechanisms. However, there is some confusion about the definition and concept [6,7,15]. The definition of the RDS used in the present paper, i.e. the step represented by the peak giving the maximum free-energy difference, is the same in principle as that used by Ray [6], who defined it as the step causing the largest change in overall rate on perturbation, although the present definition is a semiquantitative one, and can be applied only to reactions that involve a discrete RDS. Ray's definition, which can be used in many cases, is logically unimpeachable but involves an ambiguity about the mode of the perturbation. Simultaneous perturbation of the forward and reverse rate constants of a step, which comes from a change in the free-energy level of the transition state of the step (as used in the present and Ray's theory), is essential for the formulation of the RDS as a concept with practical meaning. If the rate constants were perturbed independently, all the steps in the RDZ defined by eqn. (19) would become rate-determining. The definition of the RDS with this restriction in the perturbation mode of the rate constants is useful but does not account for all usages of the concept. Various phenomena such as the effects of substrate structure, substrate concentration and pH on the overall rate have been interpreted in terms of RDS [e.g. 16] and in such cases the rate constants are not necessarily perturbed in the manner described above. At present, it is difficult to provide a unified definition of the RDS that is also applicable to such arguments. In this respect, the concept of the RDZ will partly compensate the defect in the RDS definition. All the rate constants involved in RDZ affect the overall rate equally when perturbed independently, but simultaneous changes in rate constant that can be represented by the changes in the height of the peaks or the depth of the troughs in RDZ, except those represented by the changes in the initial trough and final peak, do not affect the overall rate. This is a useful criterion for discussion of the effects of factors on the overall rate. The irregular effect of the rate-constant perturbation on the overall rate observed by Northrop [15] can be interpreted in terms of the presence or absence of a distinct RDZ and the involvement or non-involvement of the perturbed step in the RDZ. An examination of the RDZ concept also produces a cautionary note about the use of the usual

explanation of kinetic isotope effects. Kinetic isotope effects, for example those of deuterium, are often attributed to a lower zero-point energy occupied by the ground state of a deuterium-containing intermediate than that of a hydrogen-containing one and not to a lower energy of the corresponding transition state [17]. This explanation is incorrect when used beyond the simple interpretation of the lower rate of reaction of deuterated substrates. The lower zero-point energy of an intermediate does not reduce the overall rate in multistep reactions. It is necessary to assume zero-point energies that are lower to the same extent for all the intermediates and transition states, except the transition state of the RDS. In this situation, the free-energy profile becomes equivalent to one in which only the free energy of the rate-determining transition state goes up. Some properties of the RDZ have also been discussed by Ray [6] under the name of 'bottle neck'. It should also be mentioned that concepts intimately related to RDS and RDZ have been provided in metabolic control analyses [18,19]. Perturbation of the activity of a particular enzyme in order to identify the most sensitive step in a metabolic process is kinetically equivalent to perturbing a transition state in single enzyme reactions, changing both the forward and reverse rates of a step simultaneously. The critical difference between single enzyme reactions and metabolic processes is that in the former the sum of the intermediates should be equal to total enzyme concentration and the overall rate is expressed on the basis of this amount. I have previously pointed out that the recognition of the two types of RDS, one applicable to enzyme reactions and one applicable to ordinary chemical reactions or metabolic processes, is necessary for critical discussion of RDS [7].

In conclusion, a convenient procedure for relating free-energy profiles and normal enzyme kinetics has been developed. The combined use of the free-energy profiles and kinetic expressions in the present style will facilitate discussions of enzyme mechanisms. The arguments about the basic properties of free-energy profiles such as the requirement of overall stoichiometry will also be helpful in avoiding confusion in discussions based on free-energy profiles.

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