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General Mathematical Formula for Near Equilibrium Relaxation Kinetics of Basic Enzyme Reactions and its Applications to Find Conformational Selection Steps.

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Abstract

A general mathematical formula of basic enzyme reactions was derived with nearly no dependence on conditions nor assumptions on relaxation kinetic processes near equilibrium in a simple single-substrate-single-product enzyme reaction. The new formula gives precise relationships between the rate constants of the elementary reaction steps and the apparent relaxation rate constant, rather than the initial velocity that is generally used to determine enzymatic parameters according to the Michaelis-Menten theory. The present formula is shown to be complementary to the Michaelis-Menten formulae in a sense that the initial velocity and the relaxation rate constant data together could determine the enzyme-substrate dissociation constant K_{ES} , which has been usually conditionally approximated by the Michaelis constant K_M within the framework of the Michaelis-Menten formulae. We also describe relaxation kinetics of enzyme reactions that include the conformational selection processes, in which only one enzymatic conformer among a conformational ensemble can bind with either the substrate or product. The present mathematical approaches, together with numerical computation analyses, suggested that the presence of conformational selection steps in enzymatic reactions can be experimentally detected simply by enzymatic assays with catalytic amounts of enzyme.

1: Introduction

The universal mathematical formulae that are used to evaluate the physical parameters of enzymatic reactions are those given by the Michaelis-Menten theory [1]. The parameters that can be obtained by experiments according to the theory are the well known Michaelis constant K_M and the maximum velocity V_{max} , for which the relationships with rate constants at elementary enzyme reaction steps are fully established ([2] for example). Scheme 1 is an example of a simple single-substrate-single-product enzyme reaction:

The numberings in Scheme I of the rate constants may seem somewhat irregular. We preserve numbers 1, 2 etc. for other chemical steps in other more complicated schemes which will appear in consequent schemes below. In addition, in Scheme I, we denote

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interconvertible chemicals as X and Y instead of calling one chemical a substrate and the other a product. With such numberings and terminology, the Michaelis-Menten approximations [1] simplify Scheme I to either of the followings,

Scheme I_{X→Y} Scheme I_{Y→X}

and then the K_M for the $X \rightarrow Y$ and $Y \rightarrow X$ directions, termed hereby as $K_M^{X \rightarrow Y}$ and $K_M^{Y \rightarrow X}$, respectively, are given as follows [2].

$$K_M^{X \rightarrow Y} = \frac{k_4 + k_{15}}{k_3} \quad (1)$$

$$K_M^{Y \rightarrow X} = \frac{k_6 + k_{16}}{k_5} \quad (2)$$

Likewise, the $V_{\max}$ for the $X \rightarrow Y$ and $Y \rightarrow X$ directions are given by,

$$V_{\max}^{X \rightarrow Y} = k_{15} C_E^t \quad (3)$$

$$V_{\max}^{Y \rightarrow X} = k_{16} C_E^t \quad (4)$$

where C_E^t is the total concentration of enzyme. The experimental protocol that meets the Michaelis-Menten conditions is to place an enzyme in *excess* amount of its substrate and *zero* concentration of its product. In such a framework, we see that the product accumulation is linear with time for a certain period. The linear accumulation rate, i.e. the initial velocity, enables computing K_M and $V_{\max}$ [2]. Such an experimental design is applicable for isolated enzymes *in vitro*, but it may not be suitable for investigating enzymes in other circumstances, such as organs or living cells. Nowadays, it is feasible to conduct

spectroscopic measurements on concentration changes of chemicals in a single living cell under microscope. It is also possible to increase concentration of the substrate of a particular enzyme up to a certain level in a living cell by techniques like micro-injection. It is, however, quite difficult to achieve conditions for initial velocity measurements in living cells where typically the pre-existing concentration of the product of that particular enzyme is not negligible.

In contrast to the framework of Michaelis-Menten formulae, relaxation kinetic approaches can handle chemical relaxation processes, in which multiple interconvertible chemicals co-exist and change their relative concentrations until the relaxation ends up with an equilibrium state (for a review, [3]). Model schemes for enzymatic reactions have been treated on the basis of the relaxation kinetic models. Mathematical descriptions for relationships between apparent relaxation constants and rate constants of elementary reaction steps thus established are, however, generally highly dependent on specific conditions, often the relative magnitudes of the elementary rate constants. Therefore, unambiguous interpretation of the relaxation approach requires knowledge of the magnitude of the rates at each elementary step [3].

For the simplest and basic enzymatic reaction given by Scheme I, this study has attempted to build a new mathematical formula, which is: (1) able to describe the relationships between the elementary rate constants and experimentally determinable parameters in the relaxation processes near equilibrium, where initial velocity measurements are not feasible; (2) non-conditional, meaning that the formula is general regardless of the relative magnitudes of elementary rate constants; (3) made with minimum number of very safe approximations; and (4) consistent with and complementary to the Michaelis-Menten framework.

Another motivation of this study is to seek for an assay-based method for identifying enzymes that employ conformational selection (CS) modes for substrate recognition. CS is one of molecular recognition pathways and has been demonstrated for several protein-ligand systems [4–17]. In CS, multiple conformers (CS conformer ensembles) are in an equilibrium with varying degrees of ligand binding affinity. The ligand selects a particular conformer to bind, so to speak. As well as studies on induced fit (IF), another well-established molecular recognition mechanism, investigating CS processes is increasingly important in connection with practical applications, such as drug design and discovery [4–17]. To date, the standard methods to detect the presence of CS conformer ensembles is NMR spectroscopy [7,8,13]. This requires several tens of milligrams protein concentrations and hours of spectrum accumulation even on high performance NMR spectrometers. Relaxation kinetics have also been reported [15,17], although such studies have focused essentially on CS-dependent interactions of proteins and stable ligands, rather than interconverting substrate conformations.

In such circumstances, this study has also attempted to clarify whether the following simple reaction scheme which includes CS steps (Scheme II) shows relaxation kinetic aspects that are different from those seen for Scheme I and would thereby enable us to detect the presence of CS steps.

In Scheme II, enzyme conformers E^X and E^Y bind only with X and Y, respectively, and E^i is inactive towards substrate bindings. This Scheme, assuming relatively fast CS conformer conversions, when assayed according to the Michaelis-Menten framework gives an *apparent* K_M , which is a function of rate constants including those of the CS conformer conversions as given by Eq. (5) or (6) (Appendix 1).

$$K_{M_app}^{X \rightarrow Y} = \frac{k_4 + k_{15}}{k_3} \cdot \frac{k_{19}k_{22} + k_{20}k_{21} + k_{19}k_{21}}{k_{20}k_{21}} \quad (5)$$

$$K_{M_app}^{Y \rightarrow X} = \frac{k_6 + k_{16}}{k_5} \cdot \frac{k_{19}k_{22} + k_{20}k_{21} + k_{19}k_{21}}{k_{19}k_{22}} \quad (6)$$

Therefore, if the association (k_3 or k_5), dissociation (k_4 or k_6) and chemical step (k_{15} or k_{16}) rate constants are already known by independent experiments, it may be possible to identify the presence of CS steps by comparing the *intrinsic* K_M value calculated by Eq. (1) or (2) and the apparent one. Otherwise it is difficult to discriminate Scheme I and Scheme II type reactions by analysis within the Michaelis-Menten framework.

We examine whether the near equilibrium assay is more sensitive to the presence of CS processes in enzymatic reactions by the aid of our relaxation kinetic formula together with numerical computation approaches.

The paper is structured as follows: Section 3 gives the explicit formula (Eq. 30) for Scheme I that relates the relaxation rate constant to the intrinsic rate constants and the total concentrations of the enzyme and the substrates; Section 4 demonstrates the validation of Eq. (30) by computational simulations; Section 5 gives simplified forms of Eq. (30) and the validities and limitations of them; Section 6 gives a new enzymatic parameter that is complementary to the Michaelis constant, enabling to determine the exact dissociation constant between the enzyme and the substrates; Section 7 gives mathematical formulae for Scheme II together with practical ways to discriminate enzymatic reactions employing Schemes I and II experimentally; Section 8 compares the present study and other theoretical studies briefly.

2: Computational Procedures

Numerical solutions of kinetic differential equations were calculated on High Performance Computing Core of the Albert Einstein College of Medicine employing the fourth-order Runge-Kutta (RK) methods [18–21]. Home-made programs that were designed to simultaneously handle multiple kinetic differential equations were used to simulate chemical relaxation processes. The time-step-size in the RK routines was carefully tested within a range of 10^{-9} to 10^{-4} (sec) to find the best step-size that enabled saving calculation time without degrading the data quality. Other details are described in the texts or figure legends in the following sections.

3: Formula Derivation for Scheme I Reaction.

3-1: Differential Equations and Concentrations of Chemical Species at Equilibrium

Kinetic differential equations for the chemical species in Scheme I are given as follows.

$$\frac{d[X]}{dt} = -k_3[E][X] + k_4[EX] \quad (7)$$

$$\frac{d[Y]}{dt} = -k_5[E][Y] + k_6[EY] \quad (8)$$

$$\frac{d[EX]}{dt} = k_3[E][X] + k_{16}[EY] - (k_4 + k_{15}) [EX] \quad (9)$$

$$\frac{d[EY]}{dt} = k_5[E][Y] + k_{15}[EX] - (k_6 + k_{16}) [EY] \quad (10)$$

$$\frac{d[E]}{dt} = -k_3[E][X] - k_5[E][Y] + k_4[EX] + k_6 [EY] \quad (11)$$

When the Scheme I reaction resides at an equilibrium, right hand sides of Eqs. (7)–(11) equal zero. Then the fractions of X and Y at equilibrium, termed as X'_{eq} and Y'_{eq} are given by following equations (Appendix 2).

$$X'_{eq} = \frac{k_4 k_5 k_{16}}{k_4 k_5 k_{16} + k_3 k_6 k_{15}} \quad (12)$$

$$Y'_{eq} = \frac{k_3 k_6 k_{15}}{k_4 k_5 k_{16} + k_3 k_6 k_{15}} \quad (13)$$

Therefore, the absolute concentrations of X and Y at equilibrium, X_{eq} and Y_{eq} are given by

$$X_{eq} = C_L^t \frac{k_4 k_5 k_{16}}{k_4 k_5 k_{16} + k_3 k_6 k_{15}} \quad (14)$$

$$Y_{eq} = C_L^t \frac{k_3 k_6 k_{15}}{k_4 k_5 k_{16} + k_3 k_6 k_{15}} \quad (15)$$

where C_L^t denotes the sum of the total concentrations of X and Y.

3-2: Time Dependent Equations without Cross Terms for [E], [EX] and [EY] under Assay Conditions

When the total concentration of enzyme, C_E^t , is much smaller than C_L^t , we can introduce the following very safe approximation.

$$[Y] = C_L^t - [X] - [EX] - [EY] \approx C_L^t - [X] \quad (16)$$

Such an approximation further leads

$$\frac{d[Y]}{dt} = -\frac{d[X]}{dt} \quad (17).$$

Substituting expressions of Eqs. (16) and (17) for [Y] and $d[Y]/dt$ in Eq. (8), respectively, gives

$$\frac{d[Y]}{dt} = -\frac{d[X]}{dt} = -k_5[Y][E] + k_6[EY] = -k_5(C_L^t - [X])[E] + k_6[EY] \quad (18).$$

On the other hand, [EY] is given by

$$[EY] = C_E^t - [E] - [EX] \quad (19).$$

Therefore, Eq. (18) can be converted to

$$\begin{aligned} -\frac{d[X]}{dt} &= -k_5(C_L^t - [X])[E] + k_6(C_E^t - [E] - [EX]) \quad (20). \\ &= -\{k_5(C_L^t - [X]) + k_6\}[E] - k_6[EX] + k_6 C_E^t \end{aligned}$$

The right hand sides of Eqs. (7) and (20) are linear in [EX] and [E]. Thus we can obtain the explicit dependencies of [EX] and [E] on [X] and $d[X]/dt$ as follows.

$$[EX] = \frac{(k_5 - k_3)[X]\frac{d[X]}{dt} - (k_5 C_L^t + k_6)\frac{d[X]}{dt} - k_3 k_6 C_E^t [X]}{(k_4 k_5 - k_3 k_6)[X] - k_4 k_5 C_L^t - k_4 k_6} \quad (21.1)$$

and

$$[E] = \frac{(k_6 - k_4) \frac{d[X]}{dt} - k_4 k_6 C_E^t}{(k_4 k_5 - k_3 k_6)[X] - k_4 k_5 C_L^t - k_4 k_6} \quad (21.2).$$

And also, since $[EY]$ is given by Eq. (19),

$$[EY] = \frac{-(k_5 - k_3)[X] \frac{d[X]}{dt} + (k_5 C_L^t + k_4) \frac{d[X]}{dt} + k_4 k_5 C_E^t [X] - k_4 k_5 C_L^t C_E^t}{(k_4 k_5 - k_3 k_6)[X] - k_4 k_5 C_L^t - k_4 k_6} \quad (21.3).$$

3-3: Establishing Relationship between Rate Constants and X's Functions via Equality of Two Different Expressions for $d[EX]/dt$

Differentiating Eq. (21.1) with t becomes

$$\frac{d[EX]}{dt} \equiv \frac{A_1}{A_2} \quad (22)$$

where

$$\begin{aligned} A_1 = & \left\{ (k_5 - k_3) \left(\frac{d[X]}{dt} \frac{d[X]}{dt} + [X] \frac{d^2[X]}{dt^2} \right) - (k_5 C_L^t + k_6) \frac{d^2[X]}{dt^2} - k_3 k_6 C_E^t \frac{d[X]}{dt} \right\} \quad (22.1) \\ & \times \{ (k_4 k_5 - k_3 k_6)[X] - k_4 k_5 C_L^t - k_4 k_6 \} \\ & - \left\{ (k_5 - k_3)[X] \frac{d[X]}{dt} - (k_5 C_L^t + k_6) \frac{d[X]}{dt} - k_3 k_6 C_E^t [X] \right\} \times (k_4 k_5 - k_3 k_6) \frac{d[X]}{dt} \end{aligned}$$

and

$$A_2 = \{ (k_4 k_5 - k_3 k_6)[X] - k_4 k_5 C_L^t - k_4 k_6 \}^2 \quad (22.2).$$

On the other hand, the derivative $d[EX]/dt$ is also given by Eq. (9). Substituting Eqs. (21.1)–(21.3) into (9) gives

$$\begin{aligned} \frac{d[\text{EX}]}{dt} = & \frac{(k_3k_6 - k_5k_{16} + k_3k_{16} - k_4k_5 - k_5k_{15} + k_3k_{15})[\text{X}]\frac{d[\text{X}]}{dt}}{(k_4k_5 - k_3k_6)[\text{X}] - k_4k_5C_L^t - k_4k_6} \quad (23). \\ & + \frac{\{ (k_5k_{16} + k_4k_5 + k_5k_{15})C_L^t + k_4k_{16} + k_4k_6 + k_6k_{15} \} \frac{d[\text{X}]}{dt}}{(k_4k_5 - k_3k_6)[\text{X}] - k_4k_5C_L^t - k_4k_6} \\ & + \frac{(k_4k_5k_{16} + k_3k_6k_{15}) C_E^t[\text{X}] - k_4k_5k_{16}C_L^tC_E^t}{(k_4k_5 - k_3k_6)[\text{X}] - k_4k_5C_L^t - k_4k_6} \end{aligned}$$

Because the right hand sides of Eqs. (22) and (23) are equal, we find the following relations,

$$\begin{aligned} Q_1 \cdot [\text{X}]^2 \frac{d^2[\text{X}]}{dt^2} + Q_2 \cdot [\text{X}] \frac{d^2[\text{X}]}{dt^2} + Q_3 \cdot \frac{d^2[\text{X}]}{dt^2} + Q_4 \cdot \left(\frac{d[\text{X}]}{dt} \right)^2 + Q_5 \cdot \frac{d[\text{X}]}{dt} \quad (24) \\ = R_1 \cdot [\text{X}]^2 \frac{d[\text{X}]}{dt} + R_2 \cdot [\text{X}] \frac{d[\text{X}]}{dt} + R_3 \cdot \frac{d[\text{X}]}{dt} + R_4 \cdot [\text{X}]^2 + R_5 \cdot [\text{X}] + R_6 \end{aligned}$$

where

$$Q_1 = (k_5 - k_3)(k_4k_5 - k_3k_6) \quad (24.1)$$

$$Q_2 = -[(k_5 C_L^t + k_6)\{2k_4k_5 - k_3(k_4 + k_6)\}] \quad (24.2)$$

$$Q_3 = k_4(k_5C_L^t + k_6)^2 \quad (24.3)$$

$$Q_4 = k_3(k_5C_L^t + k_6)(k_4 - k_6) \quad (24.4)$$

$$Q_5 = k_3k_4k_6 C_E^t (k_5 C_L^t + k_6) \quad (24.5)$$

$$R_1 = (k_4k_5 - k_3k_6)\{k_3(k_6 + k_{15} + k_{16}) - k_5(k_4 + k_{15} + k_{16})\} \quad (24.6)$$

$$R_2 = [k_5\{2k_4k_5 - k_3(k_4 + k_6)\}(k_{15} + k_{16}) + 2(k_4^2k_5^2 - k_3k_4k_5k_6)]C_L^t \quad (24.7)$$

$$+ 2k_4k_6(k_4k_5 + k_5k_{15} - k_3k_{16} - k_3k_6) + (k_4k_5k_{16} - k_3k_6k_{15})(k_4 + k_6) \quad (24.8)$$

$$R_3 = -k_4(k_5C_L^t + k_6)\{ (k_5k_{16} + k_4k_5 + k_5k_{15})C_L^t + k_4k_{16} + k_4k_6 + k_6k_{15} \} \quad (24.9)$$

$$R_4 = (k_4k_5 - k_3k_6)(k_4k_5k_{16} + k_3k_6k_{15})C_E^t \quad (24.10)$$

$$R_5 = [\{k_3k_4k_5k_6(k_{16} - k_{15}) - 2k_4^2k_5^2k_{16}\}C_L^t - k_4k_6(k_4k_5k_{16} + k_3k_6k_{15})]C_E^t \quad (24.11)$$

$$R_6 = k_4(k_5C_L^t + k_6)k_4k_5k_{16}C_L^t C_E^t \quad (24.12).$$

3-4: Converting $[X]$, $[X]^2$, $d^2[X]/dt^2$ etc. in Eq. (24) to Functions of $d[X]/dt$ within a Near Equilibrium Range giving a Single Exponential Relaxation Rate Constant

When a reaction system in the form of scheme I relaxes within ranges close to equilibrium, the relaxation is satisfactorily described as a single exponential process, as verified by numerical computations in later sections. Then the concentration of X can be expressed as follows,

$$[X] = \delta_X \cdot e^{-kt} + X_{eq} \quad (25)$$

where δ_X is a small deviation at $t = 0$ of the concentration of X as compared to the equilibrium value X_{eq} , and k is the relaxation rate constant. Eq. (25) and associated rate constant k can be safely introduced, if we take only single exponential phases of experimental data in practical applications. Having the rate constant k in an expression for $[X]$, we are going to establish relationship between k and the elementary rate constants by substituting $[X]$ in Eq. (24) for the expression. Such a mathematical approach is somewhat unusual in a sense that it gives an explicit definition (Eq. (25)) of the apparent rate constant k in advance. In most of arithmetic formulations for kinetics of complex reaction systems, apparent rate constants are derived as mathematical solutions of simultaneous equations for rate constants of elementary reaction steps. Therefore, assigning an experimental rate constant to one of such solutions is usually conditional, depending on the relative

magnitudes of the elementary rate constants [3]. The present approach could avoid such condition dependencies by employing only one apparent rate constant k explicitly.

Differentiating Eq. (25) with respect to t gives

$$\frac{d[X]}{dt} = -k \cdot \delta_X \cdot e^{-kt} \quad (26),$$

therefore, $[X]$ is also given by

$$[X] = -\frac{1}{k} \frac{d[X]}{dt} + X_{eq} \quad (27).$$

Likewise, X 's functions $[X]^2$, $d^2[X]/dt^2$, $[X]d^2[X]/dt^2$ etc. in Eq. (24) can be expressed with $d[X]/dt$ as a single variable. Substituting X 's functions in Eq. (24) for their expressions with $d[X]/dt$ and rearranging terms yield

$$\begin{aligned} & \left(\frac{R_1}{k^2} + \frac{Q_1}{k} \right) \left(\frac{d[X]}{dt} \right)^3 - \left\{ \frac{k(2R_1 \cdot X_{eq} + R_2) - R_4}{k^2} + 2Q_1 \cdot X_{eq} + Q_2 + Q_4 \right\} \left(\frac{d[X]}{dt} \right)^2 \\ & + \left\{ R_1 \cdot X_{eq}^2 + R_2 \cdot X_{eq} + R_3 - \frac{2R_4 \cdot X_{eq} + R_5}{k} + k(Q_1 \cdot X_{eq}^2 + Q_2 \cdot X_{eq} + Q_3) + Q_5 \right\} \frac{d[X]}{dt} \\ & = 0 \end{aligned} \quad (28)$$

(Appendix 3).

3-5: Removing the Variable $d[X]/dt$ from Eq. (28) to Reach the Final Formula for the Relaxation Rate Constant k

Eq. (28) relates the relaxation rate constant k to the rate constants of chemical steps, although it still contains the time derivative $d[X]/dt$ and its second and third powers. These can be converted to constant terms upon integrating them from $t = 0$ to ∞ as follows.

$$\int_{t=0}^{t=\infty} \frac{d[X]}{dt} dt = \int_{t=0}^{t=\infty} -k \cdot \delta_X \cdot e^{-kt} dt = -\delta_X \quad (28.1)$$

$$\int_{t=0}^{t=\infty} \left(\frac{d[X]}{dt} \right)^2 dt = \int_{t=0}^{t=\infty} k^2 \cdot \delta_X^2 \cdot e^{-2kt} dt = \frac{k \cdot \delta_X^2}{2} \quad (28.2)$$

$$\int_{t=0}^{t=\infty} \left(\frac{d[X]}{dt} \right)^3 dt = \int_{t=0}^{t=\infty} -k^3 \cdot \delta_X^3 \cdot e^{-3kt} dt = -\frac{k^2 \cdot \delta_X^3}{3} \quad (28.3)$$

Therefore, integrating Eq. (28) from $t = 0$ to ∞ yields

$$\begin{aligned} & -\left(\frac{R_1 + kQ_1}{3} \right) \cdot \delta_X^3 - \left\{ \frac{k(2R_1 \cdot X_{eq} + R_2) - R_4}{k} + k(2Q_1 \cdot X_{eq} + Q_2 + Q_4) \right\} \left(\frac{\delta_X^2}{2} \right) \\ & + \left\{ R_1 \cdot X_{eq}^2 + R_2 \cdot X_{eq} + R_3 - \frac{2R_4 \cdot X_{eq} + R_5}{k} + k(Q_1 \cdot X_{eq}^2 + Q_2 \cdot X_{eq} + Q_3) + Q_5 \right\} (-\delta_X) \\ & = 0 \end{aligned}$$

(29).

Rearranging the terms gives a quadratic equation for k

$$\begin{aligned} & \left\{ \frac{Q_1}{3} \left(\frac{\delta_X}{X_{eq}} \right)^2 + \left(Q_1 + \frac{1}{2} \frac{Q_2}{X_{eq}} + \frac{1}{2} \frac{Q_4}{X_{eq}} \right) \left(\frac{\delta_X}{X_{eq}} \right) + \left(Q_1 + \frac{Q_2}{X_{eq}} + \frac{Q_3}{X_{eq}^2} \right) \right\} k^2 \quad (30). \\ & + \left\{ \frac{R_1}{3} \left(\frac{\delta_X}{X_{eq}} \right)^2 + \left(R_1 + \frac{1}{2} \frac{R_2}{X_{eq}} \right) \left(\frac{\delta_X}{X_{eq}} \right) + \left(R_1 + \frac{R_2}{X_{eq}} + \frac{R_3}{X_{eq}^2} + \frac{Q_5}{X_{eq}^2} \right) \right\} k \\ & - \left\{ \frac{1}{2} \frac{R_4}{X_{eq}} \left(\frac{\delta_X}{X_{eq}} \right) + \left(2 \frac{R_4}{X_{eq}} + \frac{R_5}{X_{eq}^2} \right) \right\} \\ & = 0 \end{aligned}$$

Accordingly,

$$k = \frac{-b - \sqrt{b^2 - 4ac}}{2a}$$

with

$$a = \frac{Q_1}{3} \left(\frac{\delta_X}{X_{eq}} \right)^2 + \left(Q_1 + \frac{1}{2} \frac{Q_2}{X_{eq}} + \frac{1}{2} \frac{Q_4}{X_{eq}} \right) \left(\frac{\delta_X}{X_{eq}} \right) + \left(Q_1 + \frac{Q_2}{X_{eq}} + \frac{Q_3}{X_{eq}^2} \right) \quad (30.1)$$

$$b = \frac{R_1}{3} \left(\frac{\delta_X}{X_{eq}} \right)^2 + \left(R_1 + \frac{1}{2} \frac{R_2}{X_{eq}} \right) \left(\frac{\delta_X}{X_{eq}} \right) + \left(R_1 + \frac{R_2}{X_{eq}} + \frac{R_3}{X_{eq}^2} + \frac{Q_5}{X_{eq}^2} \right) \quad (30.2)$$

$$c = -\frac{1}{2} \frac{R_4}{X_{eq}} \left(\frac{\delta_X}{X_{eq}} \right) - \left(2 \frac{R_4}{X_{eq}} + \frac{R_5}{X_{eq}^2} \right) \quad (30.3)$$

where the sign before the square root symbol was determined on the basis of results from numerical simulations described below.

4: Validation of Eq. (30) by Computational Methods.

Now we have a quadratic equation, Eq. (30), that gives the relaxation rate constant k as a function of rate constants of chemical steps in Scheme I. Although the equation was derived with a minimum necessary and very safe approximation ($[Y] = C_L^t - [X]$, when $C_E^t \ll C_L^t$), we tested the validity of the equation by the aid of computational practices. To do this, k was calculated for a large number of combinations of the rate constant values in two ways; one was direct computation of k according to the Eq. (30), and the other was numerical simulation of the $[X]$ changes by the Runge-Kutta (RK) methods followed by single exponential least-squares fits to determine k . It should be noted that the RK methods is a robust and standard way to solve differential equations numerically [18–21]. We calculated solutions of the Eqs. (7)–(11) simultaneously with given rate constant values to obtain reliable relaxation rate constant values.

In the present tests, the second order rate constants (k_3 and k_5) were varied from 1×10^7 to 9×10^7 ($M^{-1}s^{-1}$) with an increment of 1 in mantissa. Other rate constants were varied from 1×10^0 to 9×10^4 (s^{-1}) with the increment of 1 in both mantissa and exponent. Such settings of the ranges and increments logically generate 332150625 different combinations of the rate constant values, but not all of such combinations yield relaxation processes that reach an equilibrium within practical time range. Hence we conducted a screening on the RK simulated data, and sampled 9500 rate constant sets that made the relaxation complete within 10 to 100 sec, when C_L^t , C_E^t and δ_X were set at 1×10^{-3} , 1×10^{-6} and 5×10^{-5} molar, respectively. With such parameter values, all sets of the rate constants caused $[X]$ changes that were satisfactorily described as a single exponential process; a few examples are shown in Fig. 1, where the red dots show the simulated data, while the blue lines are results of single exponential least-squares fit to the data.

The accuracy of k values by Eq. (30) was then estimated on the basis of a fractional difference $(k - k_{RK})/k_{RK}$, where k_{RK} stands for the k values determined by the RK methods. Mean and standard error of the 9500 data of such a parameter was $-4 \times 10^{-4} \pm 1 \times 10^{-3}$. Such a very small value of the difference parameter ensures that Eq. (30) is indeed a good description for k , when C_E^t is much smaller (0.1 % in the above tests) than C_L^t .

5: Simplifying Eq. (30) to find C_E^t and C_L^t Dependencies of k .

5-1: Small δ_X as compared to X_{eq} .

Eq. (30) can be simplified under certain conditions. In its quadratic coefficient (30.1), the first and second terms include variables $(\delta_X/X_{eq})^2$ and (δ_X/X_{eq}) , respectively. Therefore, when δ_X is satisfactorily smaller than X_{eq} , the first and second terms become negligible as compared to the last term. Likewise, in the linear coefficient (30.2) and the constant (30.3), the last terms are dominant in such a condition. Then Eq. (30) is simplified as

$$\left(Q_1 + \frac{Q_2}{X_{eq}} + \frac{Q_3}{X_{eq}^2}\right)k^2 + \left(R_1 + \frac{R_2}{X_{eq}} + \frac{R_3}{X_{eq}^2} + \frac{Q_5}{X_{eq}^2}\right)k - \left(2\frac{R_4}{X_{eq}} + \frac{R_5}{X_{eq}^2}\right) = 0 \quad (31).$$

According to the definitions of R_n , Q_n and X_{eq} already given, the above equation is equivalent to as follows (Appendix 5).

$$k = \frac{-b - \sqrt{b^2 - 4ac}}{2a}$$

with

$$a = (uC_L^t + sk_6)(pC_L^t + s) \quad (31.1)$$

$$b = -w(pC_L^t + s)^2 + s^2k_3(k_5C_L^t + k_6)C_E^t \quad (31.2)$$

$$c = s^2(pC_L^t + s)C_E^t \quad (31.3)$$

where

$$p = k_3k_5(k_{15} + k_{16}) \quad (31.4)$$

$$s = k_4k_5k_{16} + k_3k_6k_{15} \quad (31.5)$$

$$u = k_3k_5(k_6k_{15} + k_4k_{16}) \quad (31.6)$$

$$w = (k_4k_6 + k_6k_{15} + k_4k_{16}) \quad (31.7)$$

5-2: Approximate solution of Eq. (31).

For the first term ($w(pC_L^t + s)^2$, termed b_1) and the second one ($s^2k_3(k_5C_L^t + k_6)C_E^t$, termed b_2) of the linear coefficient b (31.2), the highest power terms of C_L^t are $(C_L^t)^2$ and C_L^t , respectively, and their coefficients are $w p^2$ and $s^2k_3k_5C_E^t$, respectively, where the parameters $w p^2$ and $s^2k_3k_5$ have the same dimension ($M^{-4} s^{-8}$). Therefore, if there is no extreme difference in the magnitudes among the second order rate constants and also among the first order rate constants, the ratio of b_1 to b_2 is roughly $(C_L^t)^2/C_L^t C_E^t = C_L^t/C_E^t$. Because C_E^t is taken to be much smaller than C_L^t through the present formula derivations, the ratio of the two terms means that the magnitude of b_1 is much bigger than b_2 . Likewise, the magnitude of b_1 is much larger than the square root of the product of a (31.1) and c (31.3). Thus, an approximate solution of Eq. (31) is given as follows (Appendix 6).

$$k = \frac{c}{b_1} = \frac{s^2(pC_L^t + s)C_E^t}{w(pC_L^t + s)^2} = \frac{s^2}{w(pC_L^t + s)} C_E^t \quad (32)$$

Substituting Eqs. (31.4), (31.5) and (31.7) into the above gives

$$k = \frac{(k_4k_5k_{16} + k_3k_6k_{15})^2}{(k_4k_6 + k_6k_{15} + k_4k_{16})\{k_3k_5(k_{15} + k_{16})C_L^t + (k_4k_5k_{16} + k_3k_6k_{15})\}} C_E^t \quad (33).$$

This simplified form of equation indicates trends of C_E^t and C_L^t dependencies of k . The k is expected to be proportional to C_E^t , and be a linear fractional function of C_L^t .

5-3: Evaluating the Validity and Limitation of Eqs. (31) and (33).

The validities and limitations of the simplified equations Eqs. (31) and (33) were evaluated using the aforementioned 9500 sets of the rate constants. As before, C_L^t and C_E^t were set at 1×10^{-3} and 1×10^{-6} molar, respectively, on the other hand, δ_X was determined by $\delta_X = L \times X_{eq}$, where L was a positive number smaller than 1, while X_{eq} was calculated according to Eq. (14). Having the concentration parameters thus determined, we calculated k values by Eqs. (31) or (33), and also by Eq. (30) for comparison. Discrepancies between the data by the simplified equations and by Eq. (30) were estimated using a parameter $(k_{3X} - k)/k$, where k_{3X} and k are the k values calculated by either Eq. (31) or Eq. (33), and by Eq. (30), respectively. Statistics of the 9500 data sets of such a difference parameter are summarized in Table 1. As seen, Eq. (31) showed a systematic improvement of accuracy as indicated by the decrease of the difference parameter from $\sim 10^{-3}$ to $\sim 10^{-9}$, when δ_X became smaller as compared to X_{eq} by a factor from 1×10^{-1} to 1×10^{-4} . On the other hand, the approximate solution of Eq. (31), which is given by Eq. (33), showed a certain limit of its accuracy. The difference parameter stayed at $\sim 10^{-4}$ regardless of the size of δ_X tested, although the value is

still satisfactorily small. Such results of the examinations must be helpful, when the equations derived here are employed for analyses on practical enzymatic reactions.

6: New Enzymatic Parameters Available Experimentally.

Taking reciprocals of the left and right hand sides of Eq. (33) gives the following relation,

$$\begin{aligned} 1/k &= \frac{(k_4k_6 + k_6k_{15} + k_4k_{16})\{k_3k_5(k_{15} + k_{16})C_L^t + k_4k_5k_{16} + k_3k_6k_{15}\}}{(k_4k_5k_{16} + k_3k_6k_{15})^2} \frac{1}{C_E^t} \quad (34) \\ &= \frac{(k_4k_6 + k_6k_{15} + k_4k_{16})\{k_3k_5(k_{15} + k_{16})\}}{(k_4k_5k_{16} + k_3k_6k_{15})^2} \frac{1}{C_E^t} C_L^t + \frac{k_4k_6 + k_6k_{15} + k_4k_{16}}{k_4k_5k_{16} + k_3k_6k_{15}} \frac{1}{C_E^t} \end{aligned}$$

which means that an experimentally obtained $1/k$ vs C_L^t plot at constant C_E^t yields a linear line

$$1/k = \lambda_1 C_L^t + \lambda_2 \quad (35)$$

where the ratio of the intercept λ_2 to the slope λ_1 of the plot gives a parameter as follows.

$$\frac{\lambda_2}{\lambda_1} = \frac{k_4k_5k_{16} + k_3k_6k_{15}}{k_3k_5(k_{15} + k_{16})} \quad (36)$$

On the other hand, the equilibrium fractions of X and Y (X_{eq} , Y_{eq}) and are given by Eqs. (12) and (13). Therefore, the numerator $k_4k_5k_{16} + k_3k_6k_{15}$ in the expression of Eq. (36) is given as a function of either X_{eq} or Y_{eq} .

$$k_4k_5k_{16} + k_3k_6k_{15} = k_4k_5k_{16} \frac{1}{X'_{eq}} = k_3k_6k_{15} \frac{1}{Y'_{eq}} \quad (37)$$

Eqs. (36) and (37) together give relationships of the experimentally obtainable quantities λ_1 , λ_2 , X_{eq} and Y_{eq} to the elementary rate constants as follows.

$$\frac{\lambda_2}{\lambda_1} X'_{eq} = \frac{k_4k_5k_{16} + k_3k_6k_{15}}{k_3k_5(k_{15} + k_{16})} \frac{k_4k_5k_{16}}{k_4k_5k_{16} + k_3k_6k_{15}} = \frac{k_{16}}{k_3} \frac{k_4k_5k_{16}}{k_{15} + k_{16}} \quad (38X)$$

$$\frac{\lambda_2}{\lambda_1} Y'_{eq} = \frac{k_4 k_5 k_{16} + k_3 k_6 k_{15}}{k_3 k_5 (k_{15} + k_{16})} \frac{k_3 k_6 k_{15}}{k_4 k_5 k_{16} + k_3 k_6 k_{15}} = \frac{k_6 k_{15}}{k_5} \quad (38Y)$$

Here we denote the right hand sides of Eqs. (38X) and (38Y) as K_{EC} of the $X \rightarrow Y$ and $Y \rightarrow X$ reactions, respectively.

$$K_{EC}^{X \rightarrow Y} = \frac{k_4 k_{15} + k_{16}}{k_3} \quad (39X)$$

$$K_{EC}^{Y \rightarrow X} = \frac{k_6 k_{15} + k_{16}}{k_5} \quad (39Y)$$

Such coefficients resemble the K_M , which are given by Eqs. (1) and (2) for the present $X \rightarrow Y$ and $Y \rightarrow X$ reactions, respectively. Similar to the K_M , the present parameter K_{EC} can be a measure for the affinity of the enzyme with X or Y. The K_M is frequently regarded as an approximation for the substrate dissociation constant K_{ES} ($= k_3/k_4$ in the present numbering for the $X \rightarrow Y$ reaction) [2]. This approximation is, however, reliable, only if the rate constant of chemical step (k_{15}) is negligible as compared to the rate constant of substrate dissociation (k_4) [2]. On the other hand, the present parameter K_{EC} is a product of the exact K_{ES} and the fraction of the chemical rate constant ($k_{16}/(k_{15}+k_{16})$). Among them, the chemical rate constants k_{15} and k_{16} can be determined by conventional Michaelis-Menten analysis (Eqs. (3) and (4)). Therefore, when we conduct the conventional Michaelis-Menten analysis and analysis based on the present formulation on the same enzyme, we can determine the exact K_{ES} for its forward and backward reactions unconditionally.

7: Applications of the Present Formulations to Screening for Enzymes employing Conformational Selection (CS) Steps.

Here we analyze Scheme II that includes CS steps to see whether it would show differences from Scheme I with respect to the relaxation kinetics.

7-1: Formula Derivation for Enzymatic Reactions with CS Steps.

In this section, we will first establish a relaxation kinetic formula for Scheme II on the approximation that the conversions among the species E^X , E^Y and E^i are fast enough to keep an equilibrium during the relaxation processes. Such an approximation could be not always valid, and the breakdown of it would bring an important signature to discriminate Schemes I and II as we would show in the following section (Section 7-2). With this approximation, the

concentration ratios among the species E^X , E^Y and E^i are constant as represented by the following two equations from Appendix 1.

$$[E^Y] = \frac{k_{19}k_{22}}{k_{20}k_{21}} [E^X] \quad (A3)$$

$$[E^i] = \frac{k_{19}}{k_{20}} [E^X] \quad (A4)$$

A simplified equation that corresponds to Eq. (34) for Scheme I was then derived to be as follows (Appendix 7)

$$1/k = \frac{(k_4k_6 + k_6k_{15} + k_4k_{16})\{k_3k_5k_{19}k_{20}k_{21}k_{22}(k_{15} + k_{16})\}}{(k_4k_5k_{16}k_{19}k_{22} + k_3k_6k_{15}k_{20}k_{21})^2} \frac{1}{C_E^t} C_L^t \quad (40)$$

$$+ \frac{(k_4k_6 + k_6k_{15} + k_4k_{16})k_{CS}}{(k_4k_5k_{16}k_{19}k_{22} + k_3k_6k_{15}k_{20}k_{21})} \frac{1}{C_E^t}$$

where

$$k_{CS} = k_{20}k_{21} + k_{19}k_{22} + k_{19}k_{21}.$$

7-2: Actual C_L^t Dependencies of the Relaxation Rate Constant in the Scheme II Reactions.

According to Eq. (40), the relaxation rate constant k shows C_E^t and C_L^t dependencies essentially the same as those for Scheme I, being proportional to C_E^t and being a linear fractional function of C_L^t . Therefore, it will be difficult to discriminate experimentally the enzymes employing Scheme II and Scheme I by investigating their responses of k to C_E^t and C_L^t , if the interconversions among the enzyme conformers is truly fast enough as assumed.

However, when we conducted numerical RK computation to see actual C_L^t dependencies of k , we found unique features for Scheme II not seen for Scheme I. Fig. 2 compares the numerically computed $1/k$ vs C_L^t plots for Scheme I (Fig. 2A) and Scheme II (Fig. 2, B–D) reactions. For all simulations shown here, rate constants involved in both Schemes I and II (k_3 , k_4 , k_5 , k_6 , k_{15} and k_{16}) were set at values indicated in Fig. 2A. These rate constant values were chosen so that the reaction was symmetric; the association rate constants k_3 and k_5 were equal, the dissociation rate constants k_4 and k_6 were equal, and the rate constants of chemical steps k_{15} and k_{16} were also equal. In addition, the dissociation rate constants k_4 and k_6 were set to be 10 times larger than the other set of unimolecular rate constants, k_{15} and k_{16} . On the other hand, the rate constants related to the conformer conversions in Scheme II (k_{19} , k_{20} , k_{21} and k_{22}) were all kept equal, and their absolute value was varied as depicted in Fig. 2, B–D.

As seen in the results for Scheme I (Fig. 2A), numerically computed $1/k$ values (red dots) showed almost perfect agreements with the theoretical values (pink circles) that were calculated by Eq. (34), and the numerical data showed a perfect linearity (the correlation coefficient γ was exactly 1 within 7-digit accuracy) with $C_L^\dagger$. The agreement between numerical $1/k$ and theoretical ones (by Eq. (40)) became worse, when CS conformers were introduced with their conversion rate constants at $1 \times 10^5 \text{ s}^{-1}$, which was 10 times larger than k_4 and k_6 (Fig. 2B: red dots, numerical; pink open circle, theoretical data). The linearity between the numerical $1/k$ and $C_L^\dagger$ also became slightly worse ($\gamma = 0.9996$), giving an open up parabola (quadratic) characteristic. Such a reduction in the linearity became more significant, as the conversion rate constants decreased (Fig. 2, C and D).

More demonstrations of such a departure from linearity due to the presence of the CS steps are shown in Figs. 3 and 4, and Fig. S1 in Supporting Information. Among them, the reaction of Fig. 3 was symmetric as in Fig. 2, but the dissociation rate constants k_4 and k_6 were set to be 10 times smaller than the chemical step rate constants k_{15} and k_{16} . On the other hand, Fig. 4 shows results from an asymmetric reaction. In the reactions shown by Figs. 3 and 4, the conformer conversion rate constants k_{19} , k_{20} , k_{21} and k_{22} were set equal as in Fig. 2. In Fig. S1, results from three other reactions, in which the conformer conversion rate constants were partially (S1, A and B) or entirely unequal (S1C), are shown.

In all cases presented, the presence of the CS steps caused an open-up quadratic deformation to the $1/k$ vs $C_L^\dagger$ linear correlation. Such results suggested that the quadratic features could be a marker for the presence of CS steps. When the conformer conversion rates were relatively fast, the marker seemed unclear. For example, the numerical $1/k$ vs $C_L^\dagger$ data in Fig. 4B shows only weakly the quadratic features. Such weakness was, however, just simply due to the choice of the range of $C_L^\dagger$. Fig. 5B shows numerical $1/k$ data from simulations employing rate constants the same as those used for the data in Fig. 4B, but 10 times wider range of $C_L^\dagger$. In such a wider $C_L^\dagger$ range, the quadratic features appeared clearly in the $1/k$ vs $C_L^\dagger$ plot (Fig. 5B), while the perfect linearity was still seen with Scheme I for comparison (Fig. 5A).

In Fig. 5, C and D, the numerical $1/k$ data in Fig. 4B and Fig. 5B, respectively, were re-drawn together with results of quadratic least squares fits to the data. Importantly, quadratic parameters (a_0 , a_1 and a_2) were essentially unchanged between narrow (5C) and wide (5D) $C_L^\dagger$ ranges. Such a nature of the $1/k$ vs $C_L^\dagger$ correlation must be helpful in practical applications to discriminate the CS dependent response of k to $C_L^\dagger$ from other non-specific $C_L^\dagger$ effects on k , which may sometimes occur in the real enzymes in the presence of higher concentrations of substrates or products via processes, such as deformations of protein structures or dissociations of co-factors.

At the end of this section, we attempted to figure out whether complications other than the CS steps to Scheme I could also cause deformations of the $1/k$ vs $C_L^\dagger$ linear correlation. Scheme III in Supporting Information includes two additional steps to Scheme I that mimic possible unimolecular steps such as the induced fit processes. Employing the RK methods, we solved the differential equations for Scheme III and obtained numerical $1/k$ with several combinations of the relevant rate constants. Thus far we tested, resulted $1/k$ vs $C_L^\dagger$ plots

showed the perfect linearity as of Scheme I (Fig. S2 in Supporting Information). Such results are not surprising, because unimolecular processes are not affected by substrate or product concentration. Therefore, regardless of the degree of complexity, the presence of additional unimolecular steps is not expected to cause the quadratic deformations. In other words, the occurrence of the quadratic deformation is specific to the presence of enzymatic species that can bind with substrate or product, that is, the CS conformers.

7-3: Comparisons with Other Methods and the Origin of Quadratic Features.

The present study demonstrated that the near-equilibrium-relaxation-rate-constant could be a sensitive probe to detect the involvement of CS processes in enzymatic reactions. Then a question must arise whether the initial velocity of the Michaelis-Menten framework could be too. For example, the initial velocity in its reciprocal form is known to be linear with reciprocal of substrate concentration according to Lineweaver–Burk equation. Could such a linearity be affected by the presence of CS steps? To address such a question, we conducted RK calculations for Scheme II employing elementary rate constants the same as those used for Fig. 2B. To see initial velocities, $[Y]$ was set to zero at the starting point of each simulation, while $[X]$ varied in a range the same as that of C_L^t for Fig. 2B, and also in ranges 10 times larger and smaller than it. In all $[X]$ ranges tested, reciprocal initial velocity data showed the perfect linearity with $1/[X]$ (Fig. S3, A–C in Supporting Information). With CS rate constants smaller by 2-orders of magnitude, resulted reciprocal velocity data were still linear with $1/[X]$ (Fig. S3, D–F in Supporting Information). Accordingly, probing the initial velocity could not identify the presence of CS steps.

Then the next question must be why the present relaxation rate constant is sensitive to the presence of CS steps. In other words, what is the origin of the observed quadratic features in the $1/k$ vs C_L^t plots? It is obvious that the origin is in part attributed to limitations of the approximations given by Eqs. (A3) and (A4), which required the ratios among the concentrations $[E^X]$, $[E^Y]$ and $[E^I]$ be unchanged during the relaxation processes. Indeed, our RK simulations showed that $[E^Y]$ and $[E^I]$ did not completely obey Eqs. (A3) and (A4) even in cases with largest CS rate constants tested (data not shown). Therefore, certain corrections to Eqs. (A3) and (A4) are needed in order to describe the correlation between $1/k$ and C_L^t more precisely. Although complete equation derivations for such correction factors are still on going, we could find gross characters of them by the aid of our RK simulation techniques, and figured out that the term C_L^t in Eq. (40) would be replaced by a quadratic function of C_L^t , if the correction factors were introduced. Such results and complete equation derivations will be reported elsewhere.

In practical applications of the present findings to real enzymatic systems, possible experimental designs will be as simple as those used for Michaelis-Menten analysis. A great advantage is that the applications need only catalytic amounts of enzymes, as compared to several milli-grams required for physico-chemical methods that have been used to identify the presence of CS conformers, such as the NMR [7,8,13] spectroscopy or X-ray crystallography [15].

8: Comparisons of the Present Approach with Michaelis-Menten and Other Studies

The original work of Michaelis and Menten [1] made two major approximations. One was that the enzyme-substrate complex (ES) was in an equilibrium with the free forms of enzyme (E) and substrate (S) (the rapid equilibrium approximation). The other approximation assumed the backward reaction from the product (P) be negligible (the irreversible approximation). The latter approximation is valid, if the concentration [P] is negligible as compared to [S], in other words, under conditions in which the initial velocity (linear velocity) of the enzymatic reaction is seen. The rapid equilibrium approximation was later formularized in more generalized form that the concentration of the ES complex was constant over time ($d[ES]/dt = 0$) [22]. Such a formula, which is a type of the quasi-steady state approximations (QSSA), has been frequently termed as the standard QSSA (sQSSA) [23–27] in the fields of enzymology.

The irreversible approximation as above is obviously not valid in the time frame of the near equilibrium relaxation, in which S and P (X and Y in our notation) co-exist at substantial concentrations, thus we did not take it and handled the fully reversible reaction scheme (Scheme I). On the other hand, sQSSA or other types of QSSA [23–30] impose certain restrictions on the behaviors of chemical species (e.g., [ES] is fixed at a constant value within sQSSA). We did not employ QSSAs too, but made another approximation given by Eq. (6). This approximation does not explicitly impose restrictions on the behaviors of the enzymatic species, being a safe approximation as far as the enzymatic reaction is investigated for a trace amount of the enzyme in the relatively excess amounts of the substrates. Therefore, in such a condition, the time dependencies of the enzymatic species given by Eqs (21.1–3) are exact over all time range, not approximations due to restrictions by QSSAs.

Besides the present study, several theoretical studies developed mathematic formulae involving exponential rate constants, rather than the linear velocity, to describe either the rise or decay of the ES complex in wide time ranges beyond the initial velocity period [24–27,29], but such approaches were made within the irreversible approximation and by the aid of QSSAs. On the other hand, for fully reversible schemes including Scheme I and more complicated ones, the linear velocities and equilibrium constants for reaction steps were well formulated as functions of the intrinsic rate constants [31 and references cited therein], but as far as we know, our Eq. (30) formulated for the first time the exponential relaxation rate constant for the reversible reaction in a way that it is non-conditional in terms of the relative magnitudes of the intrinsic rate constants.

9: Conclusions

A novel mathematical formula was developed with a very safe approximation for describing the relaxation kinetics of the enzyme reactions. The formula provides useful basis to detect the presence of conformational selection steps in the enzyme reactions by assays using only trace amount of enzyme samples. The present study also provides a way to determine the

exact substrate dissociation constant K_{ES} of enzymes, which is frequently imperfectly approximated by the Michaelis constant K_M .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A general mathematical formula was derived for relaxation kinetic processes near equilibrium in simple single-substrate-single-product enzyme reactions.
- The present Mathematical Formula provides a way determining experimentally the enzyme-substrate dissociation constant K_{ES} , which has been usually conditionally approximated by the Michaelis constant K_M within the framework of the Michaelis-Mentene formulae.
- The formula provides useful basis to detect the presence of conformational selection steps in the enzyme reactions by assays using only trace amount of enzyme samples.

Fig. 1.

Examples of the relaxation kinetic traces of species X in Scheme I. Numerical solutions of Eqs. (7) to (11) were obtained computationally by the Runge-Kutta method to determine the concentration changes of X (red dots) with rate constant values shown in each panel. For each simulation, the initial value of $[X]$ was set $5 \times 10^{-5} \text{ M}$ larger than the value at the final equilibrium. C_L^t and C_E^t were set at 1×10^{-3} and $1 \times 10^{-6} \text{ M}$, respectively. The blue trace in each panel is a result of single exponential least-squares fit to the data.

Fig. 2.

Linearity degradation in the $1/k$ vs C_L^t correlation of the relaxation kinetics by Scheme II (B-D), as compared to the perfect linearity seen in the reaction by Scheme I (A). Numerical solutions of Eqs. (7) to (11) and (A20) to (A26) (in Appendix 7) were obtained computationally by the Runge-Kutta method to determine the $1/k$ data (red dots) in A and in B-D, respectively, at various C_L^t values. Values of the rate constants k_3 - k_{16} were common in all simulations shown here, which are indicated in A. The k_{19} - k_{22} values were varied as indicated in each of the panels B-D. C_E^t and δ_X were set at 1×10^{-7} and 1×10^{-5} M, respectively, for all data. The blue line in each panel is a result of linear least-square fit to the data, for which the linear correlation coefficient γ was obtained. Pink open circles in A and in B-D are theoretical $1/k$ values calculated by Eq. (34) and by Eq. (40), respectively.

Fig. 3.

An example of computationally observed linearity degradation in the $1/k$ vs C_L^t correlation of the relaxation kinetic reactions by Scheme II (B-D), as compared to the perfect linearity seen in the reaction by Scheme I (A). Details were the same as in Fig. 2, except that the k_3 - k_{16} values were as indicated in A.

Fig. 4.

An example of computationally observed linearity degradation in the $1/k$ vs C_L^t correlation of the relaxation kinetic reactions by Scheme II (B-D), as compared to the perfect linearity seen in the reaction by Scheme I (A). Details were the same as in Fig. 2, except that the k_3 - k_{16} values were as indicated in A.

Fig. 5.

Computational experiments for Fig. 4, A and B were further conducted in a wider range of C_L^t with the same rate constants and other conditions, and results were shown in A and B, respectively (red dots), together with results of linear least-square fits to the data (blue lines), the associated linear correlation coefficients (γ) and theoretical $1/k$ values (pink open circles) calculated by Eq. (34) (in A) or Eq. (40) (in B).

The data in B and that in Fig. 4B were re-drawn in D and C, respectively, and they were subjected to least squares fits employing a quadratic function $1/k = a_0 + a_1 C_L^t + a_2 (C_L^t)^2$. The results (blue curves) and parameters a_0 , a_1 and a_2 thus determined were as indicated in the figure.

Scheme I.

Scheme II.

Table 1:

Discrepancies in k values calculated by un-simplified Eq. 30 and simplified Eqs. 31 or 33.

$\delta_{\mathbf{x}} = L \times \mathbf{X}_{eq}, L =$	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}
Eq. 31	$(-1 \pm 3) \times 10^{-3}$	$(-1 \pm 3) \times 10^{-5}$	$(-1 \pm 3) \times 10^{-7}$	$(-1 \pm 3) \times 10^{-9}$
Eq. 33	$(-2 \pm 3) \times 10^{-3}$	$(-6 \pm 4) \times 10^{-4}$	$(-6 \pm 4) \times 10^{-4}$	$(-6 \pm 4) \times 10^{-4}$