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Measurement of Net Rate Constants from Enzyme Progress Curves without Curve Fitting

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Abstract

A method is described whereby net rate constants can be directly inferred from the progress curves of enzyme intermediates without the need for model specification, numerical analysis, curve fitting, or the steady-state approximation. Specifically, if an enzyme intermediate in an ultimately irreversible serial subsequence is perturbed from and returns back to its equilibrium state as the substrate is consumed, then its net rate constant is given by the ratio of the total substrate consumed and the area under the progress curve for the enzyme intermediate. A rigorous analysis demonstrates this result to hold independent of the complete enzymatic reaction in which the subsequence is embedded, making it broadly applicable to a very wide range of kinetic mechanisms, including those complicated by inhibition. As a theoretical consequence, it is shown that traditionally steady-state parameters such as k_{cat} , k_{cat}/K_M , and net rate constants can be expressed as limiting ratios of averages without requiring the steady-state hypothesis. Finally, a mock data set is generated for a system of contemporary interest that can serve as both an example of how the methodology would be used in practice and a proof of concept.

Graphical Abstract

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Supporting Information

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Additional mathematical details and data for replicating the mock experiments (PDF)

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The steady-state hypothesis¹ supports a useful framework for both understanding and studying the chemistry of enzymes. This is because the parameters k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ essentially reduce any applicable reaction scheme to the Michaelian model, which involves only two enzyme species (i.e., the free and the bound intermediate).¹ Net rate constants represent an abstraction introduced by Noyes² and Cleland³ that further extends the steady-state framework to include any additional known or predicted enzyme intermediates in a serial reaction sequence. Thus, the net rate constant k'_i for the i th enzyme intermediate E_i may be defined as the ratio^{3,4}

$$k'_i = \frac{\tilde{v}}{\tilde{e}_i} \quad (1)$$

where $\tilde{v}$ is the steady-state velocity and $\tilde{e}_i$ is the steady-state concentration of E_i . Though net rate constants are often regarded as a device for deriving expressions for k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ in terms of the rate constants of a specific model, they have also been recognized as useful theoretical and experimental constructs in their own right.^{a,c} In particular, like k_{cat} and $k_{\text{cat}}/K_{\text{M}}$, net rate constants are largely model agnostic and have a well-defined structure within the context of a complete model of catalysis.^{3–6} In contrast, the structure of “apparent first-order rate constants” in the context of a given catalytic model is not always clear and depends on how they are measured.^{7,8} Furthermore, catalytic commitments may be expressed in terms of net rate constants,^{9–12} and the explicit measurement of net rate constants can be useful in addressing the ambiguities inherent in k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ due to partially rate limiting steps.^{4,11–13}

However, despite the utility of net rate constants and the steady-state framework in general, it is not without its drawbacks. Foremost among these disadvantages is the steady-state hypothesis itself. For example, direct measurement of net rate constants according to eq 1 requires that enzyme intermediates be observable; however, this often requires high enzyme concentrations where a steady state may be difficult to achieve. In these cases, the fitting of numerically integrated rate equations to enzyme and substrate progress curves does not require a steady-state assumption and can provide apparent first-order rate constants but only in the context of a prespecified and appropriately conditioned kinetic model.^{8,14–17} This raises the question of whether net rate constants can be expressed in terms of non-steady-state observables that can be measured without the need for model specification, numerical analysis, and curve fitting.

In this work, such a simple representation is shown to exist for enzymatic reactions containing a subsequence of n enzyme intermediates $E_1, E_2, \dots, E_n$ having the serial structure of

^aIn this report, we distinguish between a (thermodynamic) equilibrium, where all species concentrations are unchanging, and a steady state, where only the enzyme species concentrations are necessarily unchanging.

^cNote that $(\overline{\tilde{e}_1})(t)$ is not equivalent to the product of averages given by $\tilde{x}(t)\tilde{e}_1(t)$.

where X is a substrate that binds with the first enzyme intermediate of the subsequence (i.e., E_1) and the $E_n \rightarrow E_{n+1}$ elementary reaction can always be modeled as irreversible. It should be emphasized that eq 2 is a subsequence of a complete kinetic model that describes the full enzymatic reaction. Thus, there may be additional elementary reactions and associated enzyme intermediates external to eq 2 that connect E_{n+1} back to E_1 , and these intermediates may bind and release additional substrates, products, and inhibitors in a potentially branched pathway. Furthermore, E_1 and E_{n+1} may refer to the same enzyme species, and E_1 itself may bind additional substrates, products, or inhibitors generating a branch point. It will be shown that the results developed in this report are completely insensitive to these specifications with the only requirement of the model being that the kinetics of the enzyme species $E_2, E_3, \dots, E_n$ and the substrate X be described by the subsequence of eq 2 alone. In other words, no additional assumptions of the model are necessary.

Now, suppose an enzyme-catalyzed reaction contains an embedded subsequence of intermediates described by eq 2 and is initially at thermodynamic equilibrium such that the concentrations of E_2 – E_n are all zero (see the Supporting Information). This equilibrium can be perturbed by rapidly introducing either substrate X or possibly another substrate on which the formation of E_1 depends. As X is consumed via flux through subsequence 2, the concentrations of intermediates E_2 – E_n will increase before returning back to their initial concentrations of zero as the system relaxes back to a thermodynamic equilibrium. During this time, the concentration of X will decrease from its initial value of x_0 to a new equilibrium concentration $x_0 - x_\infty$, where $0 < x_\infty < x_0$. It will be shown that if A_i is the area under the concentration progress curve for enzyme intermediate E_i ($1 < i \leq n$) acquired during this experiment, then the net rate constant for E_i will be given by

$$k'_i = \frac{\Delta x_\infty}{A_i}, \quad 1 < i \leq n \quad (3)$$

Note that x_∞ is just the absolute value of the total change in the concentration of X as the system relaxes back to equilibrium. If all of the substrate X is consumed, then $x_\infty = x_0$. However, x_∞ may be less than x_0 if the enzyme requires an additional substrate that limits the reaction or the enzyme becomes inactivated. The only requirement of relaxation is that at least some of substrate X be consumed (i.e., $x_\infty > 0$). It should be emphasized that there are no steady-state assumptions in eq 3, no requirements on the relative concentrations of the enzyme and substrate, and no curve fitting of an explicit model in the measurement of A_i , because it is just the area under the progress curve for E_i .

The following section provides a rigorous justification of the theory underlying eq 3 and a similar expression for the enzyme intermediate that binds the substrate (i.e., E_1). This then allows an interpretation of the steady-state parameters k_{cat} , k_{cat}/K_M , and net rate constants in terms of limiting ratios of averaged rates and intermediate concentrations without the need

to invoke the steady-state hypothesis. However, before wading through the abstractions to follow, the reader is encouraged to first jump to Demonstration. There, an example usage of eqs 2 and 3 is provided using mock experimental data generated from a generic model of non-heme-iron oxidase catalysis with known microscopic rate constants.

THEORY

Applicable Systems.

The theory developed here applies to all enzymatic reactions containing a subsequence of n elementary reactions described by eq 2 such that (1) E_i denotes the i th enzyme species in subsequence 2 and the enzyme species in eq 2 where $1 < i < n$ do not participate elsewhere in the enzymatic reaction, (2) in the complete enzymatic reaction, E_1 and only E_1 binds X and E_2 is the only species if any that may release X, (3) all elementary reactions $E_i \rightarrow E_j$ in subsequence 2 are governed by first-order or pseudo-first-order rate constants denoted k_{ij} with the sole exception of k_{12} , which is a second-order rate constant, (4) all “forward” rate constants $k_{i,i+1}$ where $1 \leq i < n$ are positive and all “reverse” rate constants $k_{i+1,i}$ where $1 \leq i < n$ are non-negative, and (5) the $E_n \rightarrow E_{n+1}$ elementary reaction in eq 2 can always be modeled as irreversible such that $k_{n+1,n} = 0$.

If the concentrations of species X and E_i at time t are represented by the functions x and e_i , respectively, then the preceding specification for subsequence 2 can be restated symbolically as the following system of n coupled differential equations

$$\dot{x} = -k_{12}xe_1 + k_{21}e_2 \quad (4a)$$

$$\dot{e}_2 = k_{12}xe_1 - (k_{21} + k_{23})e_2 + k_{32}e_3 \quad (4b)$$

$$\dot{e}_i = k_{i-1,i}e_{i-1} - (k_{i,i-1} + k_{i,i+1})e_i + k_{i+1,i}e_{i+1}, \quad 2 \leq i < n \quad (4c)$$

$$\dot{e}_n = k_{n-1,n}e_{n-1} - (k_{n,n+1} + k_{n,n-1})e_n \quad (4d)$$

where the overdot denotes differentiation with respect to time (e.g., $\dot{e}_i = de_i/dt$).^b It will be shown that it is unnecessary to specify any more of the kinetic model (e.g., the path connecting E_{n+1} to E_1 or whether E_1 binds an inhibitor) as long as the system of eqs 4 remains valid. Thus, an expression for $\dot{e}_1$ in eq 4 is unnecessary, and the kinetics regarding E_1 are left undefined beyond its involvement in binding X.

^bIf $n = 1$, then only eq 4a applies and k_{21} is necessarily 0. If $n = 2$, then only eqs 4a and 4b apply with k_{32} necessarily 0. Finally, if $n = 3$, then eq 4c is simply disregarded as being not meaningful. See the Supporting Information for explicit consideration of the $n = 2$ cases.

Initial Condition.

The equilibrium concentrations for all E_i in subsequence 2 where $1 < i \leq n$ are 0 independent of the enzymatic reaction in which eq 2 is embedded (see the Supporting Information for justification). However, this is not necessarily true of E_1 , X , or any other component of the complete reaction. Therefore, the initial condition of the system at time zero is described by

$$x(0) = x_0 \quad (5a)$$

$$e_i(0) = 0, 1 < i \leq n \quad (5b)$$

where x_0 is positive. The initial concentrations of all other species in the system including E_1 are non-negative, and as will become evident, a more precise definition is unnecessary.

Relaxation Hypothesis.

As the reaction system relaxes to an equilibrium from the initial condition, the functions e_i and x defined according to

$$\Delta e_i(t) = e_i(t) - e_i(0) \quad (6a)$$

$$\Delta x(t) = x(t) - x(0) \quad (6b)$$

simply represent the net change in the concentrations of E_i and X , respectively, at time t from their initial values at time zero. It can be shown¹⁸ that enzyme species $E_2, E_3, \dots, E_n$ will relax back to their equilibrium concentrations such that

$$\lim_{t \rightarrow \infty} \Delta e_i(t) = 0, 1 < i \leq n \quad (7)$$

A similar conclusion cannot be drawn for the limit

$$\Delta x_\infty = - \lim_{t \rightarrow \infty} \Delta x(t) \quad (8)$$

because the remainder of the enzymatic reaction has been left unspecified such that the behavior of e_1 and thus x as $t \rightarrow \infty$ is undefined. Therefore, the existence of the limit in eq 8 as well as the following statement is introduced as a hypothesis:

$$0 < \Delta x_\infty \leq x_0 \quad (9)$$

This simply means that at least some of substrate X must be consumed via flux through subsequence 2 as the system relaxes to equilibrium. The theory developed in the following section assumes that the enzymatic reaction in which subsequence 2 is embedded satisfies this hypothesis.

Net Rate Constants.

Cleland's recursive expression for net rate constants asserts³

$$k'_i = \frac{k_{i,i+1}k'_{i+1}}{k_{i+1,i} + k'_{i+1}} \quad (10)$$

which can be derived for serial subsequences from eq 1 using the King–Altman theory whenever a unique steady state exists.^{4,19} Now, if $n > 2$, then summing over eq 4 gives

$$\sum_{i=2}^n \dot{e}_i + \dot{x} = -k_{n,n+1}e_n, \quad n > 2 \quad (11)$$

and it follows from the fundamental theorem of calculus²⁰ that

$$\sum_{i=2}^n \Delta e_i(t) + \Delta x(t) = -k_{n,n+1} \int_0^t e_n(\tau) d\tau, \quad n > 2 \quad (12)$$

If eq 9 is satisfied, then eq 7 for all of $E_2, E_3, \dots, E_n$ implies

$$-\Delta x_\infty = -k_{n,n+1} \lim_{t \rightarrow \infty} \int_0^t e_n(\tau) d\tau, \quad n > 2 \quad (13)$$

Hence, the improper integral defined by

$$A_i = \lim_{t \rightarrow \infty} \int_0^t e_i(\tau) d\tau \quad (14)$$

exists for $i = n$ and is positive, because x_∞ and $k_{n,n+1}$ are both positive. Moreover, $k'_n = k_{n,n+1}$, because the $E_n \rightarrow E_{n,n+1}$ elementary reaction is modeled as irreversible;³ hence

$$k'_n = \frac{\Delta x_\infty}{A_n}, \quad n > 2 \quad (15)$$

Applying the same analysis to eq 4d yields

$$0 = k_{n-1,n}A_{n-1} - (k_{n,n+1} + k_{n,n-1})A_n, \quad n > 2 \quad (16)$$

such that the existence of A_n implies that $A_{n-1} > 0$ exists, and it follows from $k_{n,n+1} = k'_n$ and expression 10 that

$$k'_{n-1} = \frac{k_{n-1,n}k'_n}{k_{n,n-1} + k'_n} = \frac{\Delta x_\infty}{A_{n-1}}, \quad n > 2 \quad (17)$$

Proceeding in this manner, one can show by induction (see the Supporting Information for details) that $A_i > 0$ exists for all $i > 1$ and

$$k'_i = \frac{\Delta x_\infty}{A_i}, 1 < i \leq n \quad (3)$$

This applies to all enzyme species in eq 2 after substrate binding when $n > 2$. The special cases in which $n = 2$ are handled in the same way and worked out in the Supporting Information. Finally, if all of the substrate X is consumed upon relaxation to equilibrium, then $x_\infty = x_0$, and $k'_i = x_0/A_i$.

Note that the preceding analysis did not require that E_1 satisfy eq 7, and the improper integral A_1 does not necessarily exist. Thus, a net rate constant cannot be assigned to E_1 using eq 3. This makes sense, because such an assignment would require a steady-state concentration be defined for substrate X, which is clearly not the case here. Nevertheless, integration and taking the limit of eq 4a provide

$$-\Delta x_\infty = k_{21}A_2 - k_{12} \lim_{t \rightarrow \infty} \int_0^t x(\tau) e_1(\tau) d\tau \quad (18)$$

such that positive A_2 implies that the improper integral

$$B_1 = \lim_{t \rightarrow \infty} \int_0^t x(\tau) e_1(\tau) d\tau \quad (19)$$

exists and is positive. It then follows from eq 3 that

$$\left(\frac{k_{\text{cat}}}{k_M} \right)_X = \phi_1 \frac{k_{12}k'_2}{k_{21} + k'_2} = \phi_1 \frac{\Delta x_\infty}{B_1} \quad (20)$$

where $(k_{\text{cat}}/K_M)_X$ is the specificity constant for X when all other substrates are saturating and all product concentrations are zero and ϕ_1 is the equilibrated, fractional concentration of E_1 under those same conditions. Finally, if all of the substrate X is consumed as $t \rightarrow \infty$, then $x_\infty = x_0$ and $(k_{\text{cat}}/K_M)_X = \phi_1 x_0/B_1$.

Multiple Substrates, Products, and Inhibitors.

The key results given by eqs 3 and 20 depend only on subsequence 2 satisfying eq 4, the initial condition (eq 5), and the relaxation hypothesis (eq 9). Thus, E_1 need not return to its initial concentration as X is consumed, and no additional assumptions about those elementary reactions of the complete enzymatic reaction beyond subsequence 2 are necessary. Consequently, additional substrates, products, and inhibitors may be involved in those steps external to subsequence 2 as long as eqs 4, 5, and 9 remain valid. Furthermore, if these additional peripheral species are in sufficient excess so as to be reasonably modeled as fixed over the full time course of the reaction, then the subsequence in eq 2 may be

expanded to include the relevant elementary reactions governed by pseudo-first-order rate constants. The examples in the last section will further illustrate these points.

Limiting Ratios of Averages.

Suppose the sequence in eq 2 describes the entire enzymatic reaction such that the sole product Y dissociates irreversibly from E_n and $E_{n+1} = E_1$. One then has the idealized model shown in eq 21

In this case, $x_{\infty} = x_0$, $(k_{\text{cat}}/K_M)_X$ is just k_{cat}/K_M with $\phi_1 = 1$, and k_{cat} can be written in terms of net rate constants³ according to

$$k_{\text{cat}} = \frac{1}{\sum_{i=2}^n 1/k'_i} = \frac{x_0}{\sum_{i=2}^n A_i} \quad (22)$$

These results demonstrate that the steady-state parameters k_{cat} , k_{cat}/K_M , and the net rate constants are encoded in the decidedly non-steady-state integrals B_1 , A_2 , A_3 , etc. How is this to be understood?

All this analysis is doing is simply averaging the kinetics out over time. To see this, consider the average reaction rate over the time interval $[0, t]$, which is given by

$$\bar{v}(t) = -\frac{x(t) - x(0)}{t}, \quad t > 0 \quad (23)$$

Furthermore, the average concentration of enzyme intermediate E_i over the same interval can be expressed as

$$\bar{e}_i(t) = \frac{1}{t} \int_0^t e_i(\tau) d\tau, \quad t > 0 \quad (24)$$

The ratio of the two average quantities in eqs 23 and 24 has units of reciprocal time and may be regarded as an estimate of a net rate constant

$$k'_i \approx \frac{\bar{v}(t)}{\bar{e}_i(t)}, \quad t > 0, \quad i > 1 \quad (25)$$

The principal finding of this report indicates that this approximation becomes exact as the entire reaction time course is included (i.e., $t \rightarrow \infty$); hence

$$\lim_{t \rightarrow \infty} \frac{\bar{v}(t)}{\bar{e}_i(t)} = \frac{x_0}{A_i} = k'_i, i > 1 \quad (26)$$

The same analysis can be applied to a system in a theoretical steady state, where the reaction rate and enzyme concentrations are treated as constants. Consequently, the ratio $\bar{v}(t)/\bar{e}_i(t)$ is a constant equal to k'_i independent of time, and thus, the limit in eq 26 again reduces to k'_i . In this sense, eq 1 may be regarded as a specialization of the more general eq 26.

The steady-state parameters k_{cat} and k_{cat}/K_M have a similar representation in terms of limiting averages over the idealized model (eq 21). Thus, it can be shown (see the Supporting Information) that eq 22 rearranges to

$$k_{\text{cat}} = \lim_{t \rightarrow \infty} \frac{\bar{v}(t)}{e_0 - \bar{e}_1(t)} \quad (27)$$

for model 21, where e_0 is the fixed total enzyme concentration. Finally, the specificity constant k_{cat}/K_M can be expressed as

$$\frac{k_{\text{cat}}}{K_M} = \lim_{t \rightarrow \infty} \frac{\bar{v}(t)}{(\bar{x}\bar{e}_1)(t)} \quad (28)$$

where $(\bar{x}\bar{e}_1)(t)$ is the average product³ at t given by

$$(\bar{x}\bar{e}_1)(t) = \frac{1}{t} \int_0^t x(\tau)e_1(\tau)d\tau, t > 0 \quad (29)$$

These relationships suggest that the otherwise “steady-state” kinetic parameters k_{cat} , k_{cat}/K_M , and net rate constants may be regarded alternatively as average kinetic parameters over complete reaction time courses.

Demonstration.

The remainder of the discussion has two aims. The first is to provide a numeric demonstration that net rate constants are indeed recovered from enzyme progress curves as predicted by the theory. The second aim is to illustrate how the theory might be put to practical use. To do so, a mock experimental system is required where the underlying kinetic mechanism and rate constants are known so that progress curves can be generated for analysis. To this end, a generic non-heme-iron oxidase^{21–25} will be used, because such enzymes are of contemporary interest due to their highly reactive Fe^{IV} -oxo enzyme intermediates that produce observable progress curves.^{26–29}

Figure 1 specifies a kinetic mechanism for the generic α -ketoglutarate (αKG)-dependent non-heme-iron oxidase of the mock experiment.^{23–25} For the sake of simplicity, the αKG concentration is treated as fixed so that the “free” enzyme is the binary $[\text{E}\cdot\text{Fe}^{\text{II}}\cdot\alpha\text{KG}]$

complex (E_6) that can bind product (P) or substrate (S) to form complex $[E\cdot Fe^{II}\cdot P]$ (E_5) or $[E\cdot Fe^{II}\cdot \alpha KG\cdot S]$ (E_1), respectively. Binding of O_2 to E_1 then forms quaternary complex E_2 . Conversion of E_2 to the Fe^{IV} -oxo intermediate (E_4) is a multistep process involving oxidation of the bound αKG to succinate. This is accounted for by a dummy intermediate $[E\cdot Fe^{IV}OOKG\cdot S]$ denoted E_3 . Formation of E_3 is likely to be both fast and essentially irreversible;^{25,27,29–31} however, for the purposes of exposition, E_3 is treated here as reversibly connected to $E_1 + O_2$ and irreversibly connected to E_4 . Finally, Fe^{IV} -oxo species E_4 decomposes irreversibly concomitant with oxidation of the bound substrate to yield E_5 , which then releases product P and binds αKG to regenerate E_6 . The hypothetical rate constants assigned to each elementary reaction in Figure 1 (see also Table S1) of the mechanism were informed by published accounts of TauD and IsnB.^{27–30}

If O_2 corresponds to substrate X, then the methodology applies to subsequence 30, which includes the elementary reactions likely to be of most interest:

Given the hypothetical rate constants in Figure 1, the “true” values of the net rate constants and $(k_{cat}/K_M)_{O_2}$ can be computed using Cleland’s recursive expression (see eq 10)³

$$k'_4 = k_{45} = 10.0 s^{-1}$$

$$k'_3 = k_{34} = 30.0 s^{-1}$$

$$k'_2 = k_{23}k'_3/(k_{32} + k'_3) = 37.5 s^{-1}$$

$$(k_{cat}/K_M)_{O_2} = k_{12}k'_2/(k_{21} + k'_2) = 39.5 mM^{-1} s^{-1}$$

In the mock experiment, the initial total concentrations of the substrate (S) and enzyme will be 0.75 and 0.5 mM, respectively, prior to the addition of O_2 . Because the enzyme will equilibrate with substrate S prior to the addition of O_2 , the initial concentrations of E_6 , E_1 , and S will be ~0.32, ~0.18, and ~0.57 mM, respectively, such that if $e_i(t)$ is the concentration of E_i at time t , then

$$e_1(0) + e_6(0) = 0.5 mM$$

$$e_1(0) + s(0) = 0.75 \text{ mM}$$

$$e_6(0)s(0)/e_1(0) = k_{16}/k_{61} = 1 \text{ mM}$$

and the concentrations of all other species will be 0.0 mM in this equilibrium.

Upon rapid mixing of X (i.e., O₂) to an initial concentration (x_0) of 0.5 mM in the equilibrated enzyme solution, the system will relax back to an equilibrium state. The progress curves describing this relaxation are shown in panels A and B of Figure 2 (see the Supporting Information for simulation details). These represent the time courses an investigator might observe in a real experimental context. As expected, while E₅, E₆, and E₁ equilibrate with the product formed (i.e., P) and the residual substrate (i.e., S), substrate X is fully consumed and E₂, E₃, and E₄ return to their initial concentrations (see Figure 2B). Thus, hypothesis 9 is satisfied and $x_\infty = x_0$. The acquired concentration time course data can then be integrated using trapezoids to find the area under the respective progress curve. Thus, if $\hat{A}_4(t)$ is the area so obtained under the progress curve for E₄ up to time t as shown in Figure 2D, then one finds that the ratio $x_0/\hat{A}_4(t)$ converges to $k'_4 = k_{45} = 10 \text{ s}^{-1}$ as $t \rightarrow \infty$ (see Figure 2F), which is the result promised by eq 3. While typically only Fe^{IV}-oxo species E₄ can be readily observed, if the progress curves for E₂ and E₃ can also be recorded, then the corresponding net rate constants can be determined analogously (see Figure 2F). Likewise, if the progress curves for E₁ and X can also be observed, then $(k_{\text{cat}}/K_M)_{\text{O}_2}$ can be measured by integrating their product curve as shown in panels C and E of Figure 2 (see also eq 19).

There are several points to emphasize regarding the example analysis. First, the mock experimental system never reaches a steady state before thermodynamic equilibration. Second, numerical curve fitting was unnecessary to obtain the available kinetic parameters. Third, the analysis is insensitive to the presence of the additional substrate S and product P. Finally, the measured net rate constants are essentially model agnostic and simply reflect what can be observed. For example, the modeling of [E·Fe^{IV}OOKG·S] is likely unrealistic, and conversion of E₄ to E₅ is likely to involve multiple elementary reactions in any real non-heme-iron oxidase. Nevertheless, the values of the net rate constants are measured correctly and will apply just as well to other kinetic mechanisms with only their interpretation varying between different model specifications.

To take the demonstration one step further, suppose that substrate X had been contaminated in a 1:1 ratio with a competitive inhibitor W. While a competitive inhibitor of O₂ for non-heme-iron oxidases is arguably contrived, contamination of reagents with potential enzyme inhibitors is a practical concern for the experimentalist. In this case, the mock experiment would have included W at an initial concentration 0.5 mM, and the hypothetical kinetic model of Figure 1 would include two additional elementary reactions to account for the formation and dissociation of a dead-end complex such as E₇

Note that this modification of the kinetic mechanism is “external” to subsequence 30, because it affects only the rate equation for E_1 , leaving the system of eq 4 unchanged. Panels A and B of Figure 3 show the progress curves when $k_{17} = 50 \text{ mM}^{-1} \text{ s}^{-1}$ and $k_{71} = 10 \text{ s}^{-1}$.^d However, panels C and D of Figure 3 demonstrate that $(k_{\text{cat}}/K_M)_{\text{O}_2}$, k'_2 , k'_3 , and k'_4 are again correctly determined from the progress curve integrals despite the presence of the inhibitor. In other words, the methodology permits the experimentalist to completely ignore the fact that there was an inhibitor present in the first place.

As a final note, the previous two examples assumed that the enzyme intermediate concentrations could be observed. In practice, though, concentrations are not observed directly; rather, a signal (e.g., an ultraviolet absorbance) must be converted to a concentration via the application of a suitable multiplier (e.g., an extinction coefficient). However, integration is a linear operation, and thus, a concentration integral can just as well be obtained by application of the multiplier to the integral of the signal time course. This also means that the ratio of signal integrals will still be equal to the ratio of the concentration integrals for a given intermediate when the multiplier is not known, because the unknown multiplier will simply cancel out. This provides a route to distinguishing between enzyme mechanistic hypotheses via the comparison of changes in net rate constants (e.g., due to mutation or isotope effects), because the structure of net rate constants is well-defined.^{3,4}

CONCLUSIONS

The elementary reactions of most mechanistic interest in an enzymatic reaction often cannot be characterized without simplifying assumptions to represent the information content of the data.^{8,17} This is especially important when the progress curves are limited in availability or insensitive to external perturbations. The methodology described in this report demonstrates that the principal information available in such cases (i.e., the net rate constants) is directly encoded in the progress curve integrals and can be extracted without the need to fit a potentially ill-conditioned model. However, the methodology is limited to only a subset of the enzyme intermediates in the complete enzymatic reaction (albeit an important subset) and will provide no more information regarding binding kinetics beyond that which is already available from steady-state analyses (i.e., the k_{cat}/K_M parameters). Therefore, direct integration of progress curves should be regarded as a complement to numerical curve fitting and a convenient new tool available to the enzymologist. With regard to the general study of enzyme kinetics, however, the associated theory demonstrates that k_{cat} , k_{cat}/K_M , and net rate constants can be viewed as limiting ratios of averages providing a distinctly non-steady-state perspective on traditionally steady-state kinetic parameters.

^dIf W is an inactivator (i.e., $k_{71} = 0 \text{ s}^{-1}$), then not all of substrate X will necessarily be consumed. In this case, the total change x_{∞} in the concentration of X as the reaction mixture equilibrates is used instead of x_0 to determine the net rate constants (see eq³).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Kinetic mechanism for a generic non-heme-iron enzyme that catalyzes the oxidation of α -ketoglutarate (αKG) and a substrate S to succinate (Suc) and a product P, respectively. Rate constants are based on previously reported values for non-heme-iron enzymes such as TauD and IsnB^{27–30} with arbitrary values of similar magnitude otherwise. For the sake of simplicity, αKG is treated as a fixed species and the concentration of $[\text{E} \cdot \text{Fe}^{\text{II}}]$ is disregarded so that $[\text{E} \cdot \text{Fe}^{\text{II}} \cdot \alpha\text{KG}]$ is the “free enzyme”. Similarly, dissociation and binding of succinate are ignored. The parameters $(k_{\text{cat}}/K_{\text{M}})_{\text{X}}$, k_2' , k_3' , and k_4' reflect reaction flux from $E_1 + \text{X}$, E_2 , E_3 , and E_4 , respectively, through all “downstream” elementary reactions up to and including the first irreversible elementary reaction as shown.

Figure 2.

Mock experiment and integrated progress curve analysis based on the kinetic model in Figure 1. Initial concentrations of S, E₆, and E₁ were $s(0) = 0.57$ mM, $e_6(0) = 0.32$ mM, and $e_1(0) = 0.18$ mM, respectively, as described in the text. All other species have an initial concentration of 0.0 mM except for substrate X (i.e., O₂), which is injected at time zero to an initial concentration of $x_0 = 0.5$ mM. (A) Simulated progress curves for S, X, P, E₁, E₅, and E₆. (B) Simulated progress curves for E₂, E₃, and E₄ (initial concentrations at time zero are all 0.0 mM and removed for the semilog plot). (C) Determination of the integrals $\hat{B}_1(t)$ for the product of the E₁ and X progress curves vs time t. (D) Determination of the integrals $\hat{A}_4(t)$ for the E₄ progress curve vs time t. (E) Determination of $(k_{cat}/K_M)_X = (k_{cat}/K_M)_{O_2}$ from the integrals $\hat{B}_1(t)$. The “true” value is denoted by a broken line. (F) Determination of net rate constants k'_2 , k'_3 , and k'_4 from the integrals $\hat{A}_2(t)$, $\hat{A}_3(t)$, and $\hat{A}_4(t)$, respectively. The “true” values are denoted by broken lines.

Figure 3.

Mock experiment and integrated progress curve analysis based on the kinetic model in Figure 1 in the presence of the competitive inhibitor W against X (see eq 31 and the text for details). The initial conditions are the same with the addition of 0.5 mM W at time zero. (A) Progress curves for S, X, P, W, E₁, E₅, E₆, and E₇. (B) Progress curves for E₂, E₃, and E₄ (initial concentrations at time zero are all 0.0 mM and removed for the semilog plot). (C) Determination of $(k_{cat}/K_M)_X = (k_{cat}/K_M)_{O_2}$ from the integrals $\hat{B}_1(t)$. The "true" value is denoted by a dashed line. (D) Determination of net rate constants k'_2 , k'_3 , and k'_4 from the integrals $\hat{A}_2(t)$, $\hat{A}_3(t)$, and $\hat{A}_4(t)$, respectively. The "true" values are denoted by dashed lines.