Determination of Dissociation Constants and Specific Rate Constants of Enzyme-Substrate (or Protein-Ligand) Interactions from Rapid Reaction Kinetic Data*

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SUMMARY

Using analog simulation, the kinetic properties of the reaction

\[ A + B \rightleftharpoons C \rightleftharpoons D \]

are examined and the range of validity of popular graphical analyses analyzed.

We conclude that it is frequently possible to establish both certain rate constants and microscopic dissociation constants for this system.

The increasing availability of instruments capable of following absorbance or fluorescence changes in the millisecond time range has led in recent years to a large number of studies of ligand-protein interactions. Of particular interest to the enzymologist are cases where partial reactions in the catalytic cycle may be followed. For example, the reaction of a dehydrogenase with reduced pyridine nucleotide as well as the reaction of the enzyme-coenzyme complex with the second substrate involved in the catalytic reaction may be followed in favorable cases by absorbance or fluorimetric changes. Numerous examples of such studies are discussed in the recent monograph by Gutfreund (1). Equally suited to such studies are enzymes utilizing pyridoxal phosphate as coenzyme, where large spectrophotometric changes are associated with the Schiff base intermediates involved in the reaction mechanisms (2). The flavoproteins, with extensive spectroscopic changes associated with the different oxidation-reduction states of the flavin, as well as with enzyme-substrate intermediates, also constitute a group where much useful information has been obtained from stopped flow studies (see for example, Ref. 3).

In experiments of this kind it is frequently observed that the product of the reaction appears with accurate first order kinetics and, furthermore, that the apparent first order rate constant \( k_{obs} \) varies hyperbolically with the concentration of substrate with a limiting value which is approached asymptotically as the concentration of substrate is increased. Thus, a plot of \( 1/k_{obs} \) versus \( 1/S \) is linear with a finite intercept on the ordinate.

There are two obvious reaction schemes which will give rise to this behavior:

\[
\begin{align*}
\text{MECHANISM 1} & \\
A & \rightleftharpoons A^* \\
A^* + B & \rightarrow D \\
A + B & \rightarrow C \\
A^* + B & \rightarrow D
\end{align*}
\]

where \( A \) and \( A^* \) represent protein, \( B \) the substrate (or ligand), \( C \) an intermediate in Mechanism 2, and \( D \) the product that has a formation rate \( k_{obs} \) that is experimentally measured.

Analysis of both of these mechanisms by the steady state method shows that, in general, simple behavior is not to be found. However, in both cases, the assumption that \( k_3 \) is negligible leads to the observed hyperbolic dependence of \( k_{obs} \) on \( [B] \).

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It is often possible to discriminate between the two mechanisms on the basis of the context of the experiment. In addition, there are several experimental criteria which may be employed. First, the limiting rate at high [B] observed in Mechanism 1 is $k_l$, the isomerization of A to $A^*$; this process is independent of the nature of B. Thus, the demonstration that the limiting rate varies with different substrates identifies Mechanism 2 as the relevant mechanism. This criterion has been used to support a mechanism for the reduction of cytochrome c which requires the isomerization of A to $A^*$; this process is independent of the protein whereas the second mechanism would exhibit an instantaneous change associated with the conversion of A → C followed by the slower conversion of C → D. In addition, provided the reactions are irreversible, the form of the slow absorbance changes observed in Mechanism 1 should be the same for stoichiometric $B$ for Mechanism 1 and an increasing function of $[B]$ for Mechanism 2 (6).

The available evidence suggests that Mechanism 2 is very common; examples can be found with flavoproteins (7–9), pyridoxal enzymes (10), DPN requiring enzymes (11, 12), and heme-proteins (13, 14). In these cases and in others proceeding via Mechanism 2, the dissociation constant for the intermediate C, $K_d = k_3/k_4$, is obviously a valuable piece of data. There presently exists some confusion regarding the conditions under which estimates of $K_d$ from rapid reactions are valid.

This has led us to examine this mechanism under the restrictions imposed by the steady state and equilibrium methods and to test the range of validity of the equations so obtained using an analog computer. We believe the conclusions of our analysis to be of sufficient general interest to merit publication.

Although this problem has been partially treated by others or is intuitively obvious by analogy to the Michaelis-Menten and Briggs-Haldane derivations, the unified treatment presented here should be of value in future rapid reactions and chemical modification studies.

**METHODS**

Analog simulation was carried out with an Applied Dynamics AD 2-64 PD computer at the University of Michigan Simulation Center using standard techniques (15). Solutions were recorded on graph paper and further manipulated manually.

**RESULTS**

Application of the steady state condition to the species C of Mechanism 2 gives (see Appendix):

$$k_{\text{obs}} = \frac{k_1 [B] (k_3 + k_4)}{k_1 [B] + k_2 + k_3}$$ (1)

Requiring $k_2 \gg k_4$ reduces the expression to:

$$k_{\text{obs}} = \frac{k_1 k_3 [B]}{k_1 [B] + k_2 + k_3}$$ (2)

$$k_{\text{obs}} = \frac{k_1 k_3 [B]}{k_1 [B] + k_2}$$ (3)

Thus a plot of $1/k_{\text{obs}}$ versus $1/[B]$ is linear with intercept $1/k_3$; however, the slope is either $(k_3 + k_4)/k_3k_4$ or $k_3/k_4$ depending upon whether the steady state or equilibrium assumption is more appropriate. In the latter case, the ratio of slope to intercept yields $K_d$.

Figs. 1 and 2 illustrate some typical results obtained from simulations under a variety of conditions. In the two examples shown in Fig. 1, $k_3$ was set at $2 \text{s}^{-1}$ and $k_2$ to either $20 \text{s}^{-1}$ (Case I) or $200 \text{s}^{-1}$ (Case II), i.e. the equilibrium condition was imposed. In both instances the intercept of the double reciprocal plot gives the correct value for $1/k_3$ (0.5 s). Furthermore, the

**Fig. 1.** Reciprocal plot of the observed first order rate constant as a function of the concentration of B. Data obtained from analog computer studies, with the rate constants shown.
ratios of slope to intercept are $10^{-4}\text{ M}$ and $10^{-8}\text{ M}$ for I and II, respectively, in exact agreement with their dissociation constants $(k_k/k_j)$.

It is conventional to state that the steady state approximation is valid only if the concentration of any intermediate, such as $C$, be very small. From Fig. 1 it follows that this is not a necessary condition because the equilibrium equation (and hence the steady state equation) is obeyed when $k_3[B] >> k_2$ so that $[C]$ in fact approaches $[A]$.

When $k_3$ is much greater than $k_1 + k_2[B]$ one again observes a linear dependence of $1/k_{obs}$ on $1/[B]$ with a $y$ intercept of $1/k_3$ and a slope which asymptotes to the limiting value $1/k_1$ as the combination of $A$ and $B$ becomes the rate limiting step (Fig. 2). This is the intuitive result and is consistent with Equation 1:

$$\frac{1}{k_{obs}} = \frac{1}{k_3} + \frac{1}{k_1} \cdot \frac{1}{[B]}$$

The increasing value for $k_3$ results in a continually decreasing value for the intercept which, at large $k_3$, becomes indistinguishable from the origin. Careful measurements at high substrate concentration are thus required to differentiate between this situation and the possible existence of a simple second order reaction with $k'_3 << k'_1$ (see below).

When $k_3$ is comparable to $k_1$ (Fig. 2), one observes a nonlinear dependence of $1/k_{obs}$ on $1/[B]$, and it would seem that under these conditions neither the steady state nor equilibrium approximations are valid. In general, the rate of production of $D$ should be the difference between two exponentials (16) and plots of log $[D]$ versus time should be linear only after a significant initial lag phase. This is confirmed by the computer simulation.\footnote{The conditions necessary for a significant lag phase can be deduced from the full solution to Mechanism 2 (16). It is that the ratio $(p + q)/(p - q)$ lies in the range 2 to 20, where $p = (k_3[A] + k_1 + k_3 + k_4)$ and $q = (p - 4(k_1[A])k_2 + k_3k_4 + k_1(A[k_4])^4$. The lower limit is set by the ability to discriminate between the time dependence of two exponential terms and the upper limit by the requirement that both exponentials have a finite amplitude. For the case in Fig. 2 where $k_4 = 20\text{ s}^{-1}$ and $1/[B] = 20,000$ to 40,000, the computer simulation does appear to obey this law at $[B] > 1/20,000$ when the second order step is approximately at equilibrium. At lower concentrations of $B$, however, the computer solutions do not conform to any simple expression.}

When $k_3$ can be determined in the above manner, $k_3$ and $K_d$ may be obtained by secondary treatment of the experimental data. This is done by plotting $1/(k_{obs} - k_4)$ versus $1/[B]$ as shown in Fig. 4 for the two cases of Fig. 3. The reciprocal plot is equivalent to that of Fig. 1, the intercept yielding $1/k_3$ and the slope/intercept yielding $k_d$. The theoretical justification for this is readily seen from the definition of:

$$k_{obs} = k_3\left(\frac{K_d}{[B]} + 1\right) + k_4$$

Thus:

$$\frac{1}{k_{obs} - k_4} = \frac{1}{k_3} + \frac{K_d}{k_3}\frac{1}{[B]}$$

FIG. 2. Reciprocal plot similar to Fig. 1, showing the tendency for the plot to become that of a second order reaction when the concentration of $B$ is lowered to that of $A$.

FIG. 3. Reciprocal plot similar to that of Fig. 1, but with a finite value of $k_3$. Data from analog computer studies, with the rate constants shown. Note that the extrapolated $1/k_{obs}$ values equal $1/(k_3 + k_4)$ and that at low concentrations of $B$ a limiting value of $k_{obs}$ equal to $k_4$ is obtained. See also Fig. 6 for a graphical method of determining $k_4$. However, for $k_3 = k_4$ the steady state solution reduces to:

$$\frac{1}{k_{obs}} = \frac{1}{k_3} + \frac{2}{k_1}\frac{1}{[B]}$$

and the analog computer solution does appear to obey this law at $[B] > 1/20,000$ when the second order step is approximately at equilibrium. At lower concentrations of $B$, however, the computer solutions do not conform to any simple expression.

Cases where $k_4$ Is Finite—As predicted from Equations 1 and 2, when $k_4$ is finite, the reciprocal plots are markedly nonlinear, except at high concentrations of $B$, when they extrapolate to a value of $1/k_{obs}$ equal to $1/(k_3 + k_4)$. This is illustrated in Fig. 3 for the two situations where $k_4$ is $2\text{ s}^{-1}$ and is set either to 2 or 20 $\text{s}^{-1}$. Comparison of this figure with Figs. 1 and 2 reveals an important diagnostic distinction between the cases where $k_4$ is zero and finite. In the former case, when the range of concentration of $B$ is extended to include values close to that of $A$, the reciprocal plot remains linear or curves up. On the other hand, when $k_4$ is finite, the reciprocal plot curves down and the values of $1/k_{obs}$ extrapolate to a value equal to $1/k_4$ at low concentrations of $B$. This situation arises from the fact that at low concentrations of $B$ the expression for $k_{obs}$ (Equation 2) asymptotes to $k_4$.

When $k_4$ can be determined in the above manner, $k_3$ and $K_d$ may be obtained by secondary treatment of the experimental data. This is done by plotting $1/(k_{obs} - k_4)$ versus $1/[B]$ as shown in Fig. 4 for the two cases of Fig. 3. The reciprocal plot is equivalent to that of Fig. 1, the intercept yielding $1/k_3$ and the slope/intercept yielding $k_d$. The theoretical justification for this is readily seen from the definition of:

$$k_{obs} = k_3\left(\frac{K_d}{[B]} + 1\right) + k_4$$

Thus:

$$\frac{1}{k_{obs} - k_4} = \frac{1}{k_3} + \frac{K_d}{k_3}\frac{1}{[B]}$$
FIG. 4. Secondary treatment of the data of Fig. 3, showing a linear double reciprocal plot when the determined value of \( k_4 \) is subtracted from \( k_{obs} \).

FIG. 5. Reciprocal plot of the fraction of \( D \) formed at equilibrium as a function of the concentration of \( B \). The endpoints were obtained from the analog computer runs of Fig. 3; the values of the rate constants used are given in Fig. 3.

A second criterion which can be employed experimentally to distinguish between the cases when \( k_4 \) is zero or finite is the fraction of the theoretical concentration of \( D \) observed as the concentration of \( B \) is varied. When \( k_4 \) is zero, the full theoretical concentration of \( D \) is always reached in the reaction; when \( k_4 \) is finite the fraction of \( D \) formed at equilibrium is governed by the relative values of \( k_2 \) and \( k_4 \) and the concentration of \( B \). Fig. 5 shows this point for the two cases of Fig. 3. When \( k_2 = k_4 \), the equilibrium concentration of \( D \) formed at infinite concentration of \( B \) (obtained by extrapolation of the reciprocal plot) is 0.5, as expected. Similarly, when \( k_2 \) is 10 \( k_4 \), the intercept value corresponds to the expected value of 0.91.

From the above discussion it is evident that observation of the extent of completion of a given reaction as the concentration of one of the reagents is varied, and suitable treatment of the data are valuable ways of determining if an equilibrium process exists and provide independent information on the relative values of the forward and reverse rate constants, \( k_4 \) and \( k_{-4} \), which should be consistent with the values of these rate constants obtained as shown in Figs. 3 and 4.

It is instructive to compare the behavior of the simple reversible equilibrium:

\[
A + B \xrightarrow{k_{-1}} D
\]

for which \( k_{obs} = k_1 [B] + k_2 \), and Mechanism 2 (Fig. 6). In the case of a simple reversible equilibrium, \( k_{obs} \) is a linear function of \([B]\) with slope \( k_1 \) and intercept at zero \([B]\) = \( k_2 \). In contrast, such plots are nonlinear for the two-step Mechanism 2 as can be seen from Equation 2: at high \([B]\), the limiting rate is the sum of the rate constants of the second equilibrium, \( k_3 + k_4 \), and at zero \([B]\), the limiting rate is \( k_2 \). Thus, this plot is not only useful in distinguishing between one- and two-step equilibrium processes, but, by extrapolation, also provides numerical values for \( k_{1} \) or \( k_{4} \), as the case may be.

**DISCUSSION AND CONCLUSIONS**

As considered in the introductory section, there are a number of methods which can be applied to discriminate between the two possible mechanisms discussed. We shall confine ourselves to consideration of the commonly encountered Mechanism 2, the two-step equilibrium process involving the formation of a protein-ligand complex followed by an isomerization (or further reaction). This mechanism is readily differentiated from a simple one-step equilibrium reaction by the graphical method shown in Fig. 6, which also provides an accurate method for the determination of \( k_{1} \) or \( k_{4} \). If a finite value of \( k_4 \) is found, this may be used in secondary plots such as those of Fig. 4 to determine the value of \( k_2 \), and to provide a linear slope in such reciprocal plots.

Of great practical interest is the question of whether suitable plots of kinetic data can be used to obtain valid estimates of the dissociation constant of the primary protein-ligand binding. Depending on whether steady state (Equation 3) or equilibrium
(Equation 4) conditions apply, the ratio of slope to intercept of reciprocal plots of the type shown in Figs. 1 and 4 is respectively \((k_t + k_3)/k_1\) or \(k_2\). Fortunately there are experimental criteria which can be used to differentiate between these two cases. When equilibrium conditions apply, i.e. \(k_3 \gg k_1\), the semilogarithmic plots used to determine \(k_{obs}\) are perfectly linear from zero time of reaction, and reciprocal plots such as those of Fig. 1 are linear over a wide range of \([B]\). On the other hand, when the steady state conditions apply, i.e. \(k_3 \gg k_1\), the primary semilogarithmic plots show significant lags before becoming linear. Furthermore, as illustrated in Fig. 2, the reciprocal plots are quite distinctive. When \(k_t = k_3\), the reciprocal plot curves upward markedly as \([B]\) is lowered, but a finite intercept is readily obtained (equal to \(k_3\)). When \(k_3 \gg k_1\), the reciprocal plot again approximates closely to a straight line, but extrapolates towards the origin, and, as already stated, it requires careful measurements at high concentrations of \([B]\) to distinguish this possibility from an irreversible second order reaction.

Hence it can be concluded that if the primary semilogarithmic plots used to obtain \(k_{obs}\) show no lag phase and if the secondary reciprocal plots are linear and give a finite intercept, then the ratio of slope to intercept in such plots yields the dissociation constant, \(K_a = k_2/k_1\). Thus, with sufficient data collection over a wide range of substrate concentration, and suitable graphical analysis, it is possible to determine from stopped flow measurements not only the rate constants \(k_a\) and \(k_q\), but also the otherwise difficult to obtain ratio, \(k_3/k_1\).

APPENDIX

Steady State Solution (Equation 1):

\[A + B \xrightarrow{k_1} C \xrightarrow{k_3} D\]

Applying the steady-state condition to \(C\):

\[k_1[A][B] + k_4[C] = (k_2 + k_4)[C]\]

\[\lim\limits_{t \to \infty} (C) = (A) = [A]_{eq}\]

\[k_2[B][C] = (k_2 + k_3)[C] = (k_2 + k_3)[D]\]

\[\text{rate} = \frac{k_2[B][C]}{k_2[B] + k_3}\]

\[\frac{d[D]}{dt} = k_2[C] - k_u[D]\]

\[\frac{d[C]}{dt} = -k_2[C] + k_u[D]\]

\[k_2[B][C] = (k_2 + k_3)[D]\]

\[\text{rate} = \frac{k_2[B][C]}{k_2[B] + k_3}\]

\[\text{rate} = \frac{k_2[B][C]}{k_2[B] + k_3}\]

The equilibrium condition requires \(k_t \gg k_3\); \(k_3\) then drops out of the denominator.

REFERENCES

Determination of dissociation constants and specific rate constants of enzyme-substrate (or protein-ligand) interactions from rapid reaction kinetic data.

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