CHAPTER 14

PUTTING KINETIC PRINCIPLES INTO PRACTICE

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The overall goal of efforts to characterize enzymes is to document their molecular and kinetic properties. Regardless of the exact mechanism of an enzyme reaction, a kinetic characterization often makes use of the simple Michaelis–Menten model:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \tag{14.1}$$

the ultimate objective being to provide estimates of the kinetic constants, K_m and $V_{\max}$, under a defined set of conditions:

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{14.2}$$

$$V_{\max} = k_2[\mathrm{E}_T] \tag{14.3}$$

Once these kinetic constants are determined, the specificity constant for various substrates and under defined conditions can be obtained as

$$\frac{V_{\max}}{K_m} \propto \frac{k_{\mathrm{cat}}}{K_m} \tag{14.4}$$

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Since significant meaning is placed on these measured constants and parameters, it is important that they be determined accurately and unambiguously. It is also important that the reader or practitioner in the field of enzymology be able to assess if the measurement of these parameters is reliable. Furthermore, since enzyme behavior is often modeled as Michaelis–Menten (hyperbolic) kinetics, it seems reasonable that interpretations of observations should be made in the context of the Michaelis–Menten model. In some cases, alternative explanations for enzyme kinetic behavior may be appropriate and one may be inclined to select one interpretation over another (preferably based on a kinetic analysis, although too often this is done on intuition).

The purpose of this chapter is to illustrate some simple approaches to surveying the soundness of newly gathered or published information on enzyme kinetic characterization. This is intended to orient the developing enzymologist working in this field, as well guide those assessing literature reports on enzyme kinetic characterization. Fictitious examples have been constructed for this purpose, although they have been inspired by actual reports in the scientific literature encountered by this author. These specific examples will be used to illustrate putting simple kinetic principles to practice in an effort to draw the appropriate conclusions from enzyme kinetic data (and avoid reliance on one's intuition). Each of the following sections is titled in the form of a question, and these questions represent the most basic types of issues that one should consider upon reviewing enzyme kinetic data, whether it is one's own or has been generated by the studies of others.

14.1 WERE INITIAL VELOCITIES MEASURED?

Perhaps the most elementary consideration that should be satisfied is that the measured rates of enzyme reactions under all conditions represent *initial velocities* (v_0). The indication that *initial rates* or *linear rates* were measured are other ways to convey that this standard of experimentation has been met. One of the original stipulations of the general applicability of the Michaelis–Menten model (as well as many others) is that $d[S_0]/dt \approx 0$ during the time period over which the rate of product formation is measured. Thus, the measured reaction rate is representative of that taking place initially at the $[S_0]$ selected. This condition is especially important at low $[S_0]$ values, where reaction rates are nearly first order with respect to $[S_0]$. In practice, up to 5 to 10% depletion of $[S_0]$ can be tolerated over the time frame used to assay [P] for the purpose of determining reaction rates, because error caused by normal experimental

variance may exceed any systematic error brought about by this degree of consumption of $[S_0]$ during the assay period.

Continuous assay procedures facilitate estimation of initial rates since the opportunity exists to linearize the initial portion of the reaction progress curve (Fig. 14.1). In contrast, the fixed-point assay, where the reaction or assay is quenched at a preselected interval(s) to allow for product measurement, requires greater care and vigilance to ensure that an estimation of initial velocity was obtained ($d[P]/dt$ must be linear during the entire assay period). Using the data in Fig. 14.1 as an example, a fixed-point assay interval of 10+ minutes would not provide for an estimate of initial velocity, whereas intervals of 6 minutes or less would.

Occasionally, fixed-point assays on the order of hours are encountered in published reports, and in these cases the reader should look very carefully and critically for assurances that measured reaction rates were linear. This author has even encountered reports where it was stated to the effect that "... reaction rates were linear and $[S_0]$ depletion was limited to 30% in all cases." Such a statement should be treated with great skepticism, since in this scenario the greatest degree of $[S_0]$ depletion would almost certainly occur at the low $[S_0]$ range tested, where the rates would most quickly deviate from linearity. It would also defy kinetic principles that reaction rates would be linear at $[S_0] \ll K_m$ for the period of time in which 30% depletion of $[S_0]$ occurred.

What could possibly go wrong if the measurement of linear rates was not assured? Well, an example has been provided to illustrate that it could mean the difference between falsely concluding that an enzyme reaction is allosteric (cooperative) and not correctly concluding that it behaves according to the simpler Michaelis–Menten model (Allison and Purich, 1979, Fig. 2). The reader is encouraged to peruse this reference for a

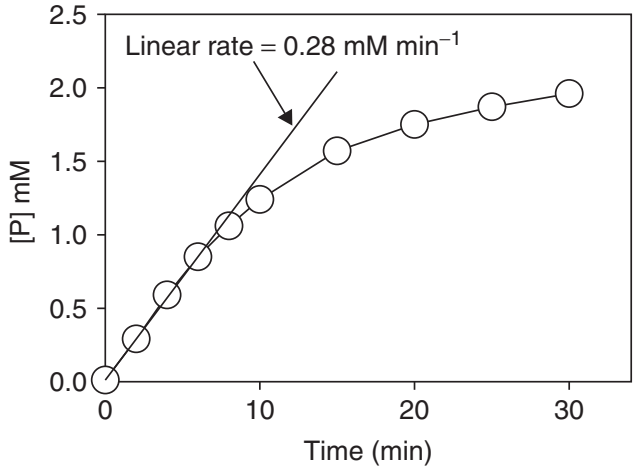

Figure 14.1. Enzyme reaction progress curve and estimation of initial velocity.

refresher on the considerations to be made in measuring *initial velocities*, which in those authors' words "... is of prime importance for achieving a detailed and faithful analysis of any enzyme."

14.2 DOES THE MICHAELIS–MENTEN MODEL FIT?

Perhaps the second most elementary (and very common) consideration regarding the kinetic profiling of an enzyme reaction is to assess whether or not it can be fitted to the Michaelis–Menten model. This assessment is not always taken as seriously as it should. Rather than truly *assess* whether or not the data conform to a Michaelis–Menten model, it is often simply stated (or blindly assumed) that they do, and various linear transformations are conducted to arrive at estimations of the kinetic constants K_m and $V_{\max}$.

Consider the data presented in Fig. 14.2, where an accompanying comment may very well be something like "... the response of enzyme activity to increasing $[S_0]$ was hyperbolic." The inset of Fig. 14.2 also illustrates a common and almost reflexive practice to transform these original data to a linear plot, often with quite "unconventional" methods for linearizing the transformed data. (The curvature to the data points in the inset appears to have been ignored, and although there are proper data weighting procedures for this specific linear plot, they appear seldom to have been evoked.) The double-reciprocal (Lineweaver–Burke) plot is the most often selected linear transform [despite repeated cautions that it is the least trustworthy of the linear plots most often considered (Henderson, 1978; Fukuwaka et al., 1985)].

Although the data in Fig. 14.2 may appear to be visually consistent with a rectangular hyperbola pattern (Michaelis–Menten model), it is a rather simple matter to test the observed data for fit to the Michaelis–Menten

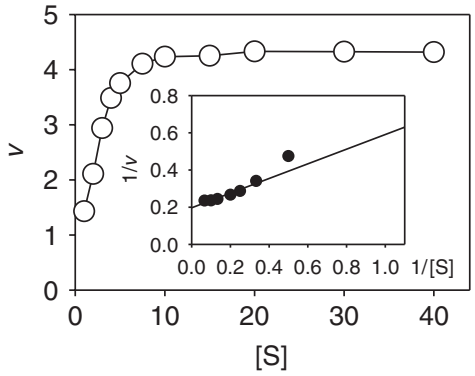

Figure 14.2. Enzyme rate data and transformation to double-reciprocal plot (inset).

model (although this is not done often enough). Taking the same data in Fig. 14.2 and imposing the rectangular hyperbola function on it,

$$y = \frac{ax}{b + x} \tag{14.5}$$

where y is the velocity, x represents $[S_0]$, a represents V_{max}, and b represents K_m, yields the boldface line in Fig. 14.3. It is clear that there is a systematic deviation of the data from the model that is readily apparent at the high- and medium-range $[S_0]$ tested. The significance of this analysis is twofold:

1. The kinetics of the enzyme reaction are more complicated than a Michaelis–Menten model can accommodate (further diagnostic tests, such as the use of the Hill plot, may reveal allosteric behavior or cooperativity as a kinetic characteristic).
2. The estimation and discussion of K_m (the Michaelis constant) may be irrelevant because K_m is a constant defined by (and confined within) use of the Michaelis–Menten model (hyperbolic kinetics) in the first place.

Different kinetic models have different conventions, and in the case of cooperative enzyme kinetic behavior, the term $K_{0.5}$ is used in a sense analogous to K_m for hyperbolic enzymes. In fact, transforming the original data in Fig. 14.2 to a Hill plot,

$$\log \frac{v}{V_{max} - v} = n \log[S] - \log K' \tag{14.6}$$

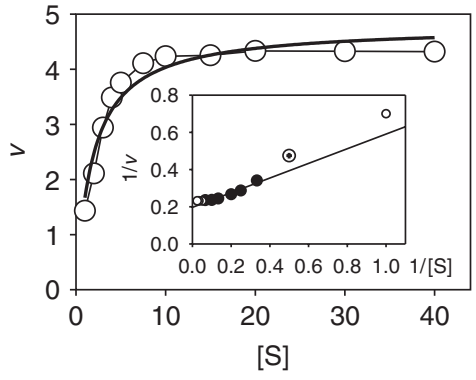

Figure 14.3. Enzyme rate data from Fig. 14.2, with predicted hyperbolic kinetics pattern (bold curve) superimposed. Inset shows data appearing in linear plot in Fig. 14.2 inset (●, ⦿), as well as that not appearing in Fig. 14.2 inset (○).

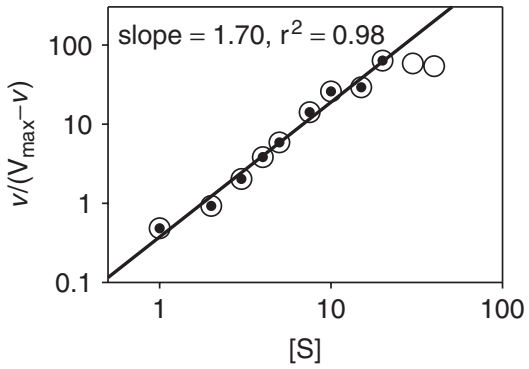

Figure 14.4. Transformation of the enzyme rate data in Fig. 14.2 to a Hill plot. Points appearing as (○) were not included in the regression analysis.

where K' is a modified intrinsic dissociation constant and n is the apparent number of enzyme subunits (and slope on the Hill plot), yields a linear region (Fig. 14.4) for the most meaningful portion of the curve in Fig. 14.2. This plot is indicative of a cooperative enzyme with two apparent subunits and a K' (or $K_{0.5}$) value of 1.8 mM (the deviation from the linear plot at the high [S] value could be caused by a cofactor becoming limiting in the assay, among other reasons).

For the discerning reader, a closer examination of the Fig. 14.2 inset, and comparison of the axis values (1/[S]) with those ([S]) of the original data set, reveals that only a subset of the original velocity versus [S_0] data set is used to construct the linear plot (both high and low [S_0] points on the linear plot are omitted). This appears to be a classic case of imposing a model on a data set rather than using the data set to direct selection of the appropriate model for enzyme kinetic behavior. Figure 14.3 (inset) shows all of the original data transformed to the linear plot, and a systematic departure from linearity is clearly evident.

14.3 WHAT DOES THE ORIGINAL [S] VERSUS VELOCITY PLOT LOOK LIKE?

From the preceding discussion it should be evident that perhaps the most important and insightful data set on enzyme kinetic behavior is the original velocity versus [S_0] plot. However, it seems more often than not that this relationship is presented as a linear plot and not as original, nontransformed data. This approach may serve to cloud one's vision instead of offering insight into enzyme kinetic behavior [see Klotz (1982) for an example of diagnosing flawed receptor/binding analysis].

As an example, consider the findings reported in Fig. 14.5 regarding the nature of inhibition of an enzyme reaction. At increasing concentrations

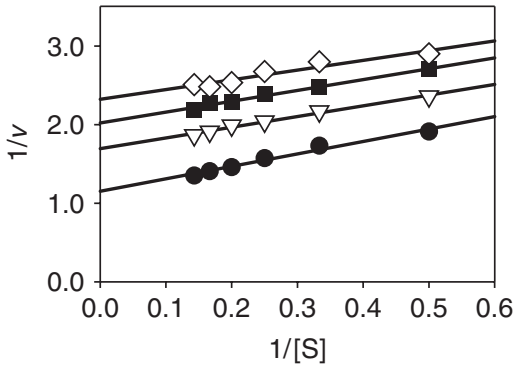

Figure 14.5. Double-reciprocal plot of enzyme rate data for assays done in the absence of inhibitor (●), and at progressively increasing levels of an inhibitor (▽, ■, ◊).

of inhibitor [I], the transformed velocity versus [S_0] plots for noninhibited and inhibited reactions display the classical pattern of uncompetitive inhibition, diagnosed as parallel plots on this linear plot for reactions inhibited by increasing levels of [I]. This data set would be used to estimate both K_m and K_I as a kinetic characterization of the inhibited enzyme reaction.

However, a closer inspection of the linear plot reveals that a very narrow range of [S_0] of only 2 to 7 mM was used for these studies. Reverting the data back to the original coordinates of velocity versus [S_0], it is also evident that the range of [S_0] used was $\geq K_m$, creating a bias in the data set where velocity is becoming independent of [S_0] (Fig. 14.6). If the data points encompassing the "missing" [S_0] range are filled in, predicted by nonlinear regression plots derived from the original data, it is clear that the range of K_m values calculated (0.56 to 1.49 mM) is rather narrow. This limited data set that does little to define or resolve the curvature of these plots, and consequently the study is not reliable or sufficiently conclusive. Finally, and to put this particular data set into a broader context, the conclusion that uncompetitive inhibition occurs should be immediately

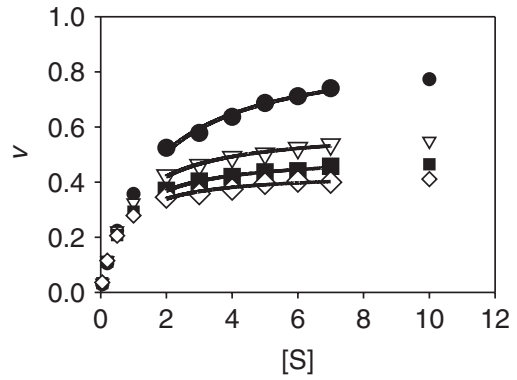

Figure 14.6. Transformation of enzyme rate data in Fig. 14.5 to a conventional velocity versus [S] plot (symbols are the same as in Fig. 14.5).

scrutinized because it is extremely rare (Segel, 1975; Cornish-Bowden, 1986). Certainly, a more compelling and persuasive data set than that in Figs. 14.5 and 14.6 would be required to support the conclusion that a rare kinetic property was discovered for a particular enzyme.

14.4 WAS THE APPROPRIATE [S] RANGE USED?

As an extension of some of the issues raised in Section 14.3, it is universally accepted that when using traditional approaches to kinetic analysis, a range of $[S_0]$ must be used to obtain reliable estimates of K_m and V_{max} (Segel, 1975; Whitaker, 1994). A range of $[S_0]$ of 0.3 to $3K_m$ (or better yet, 0.1 to $10K_m$, solubility permitting) for the purpose of estimating K_m and V_{max} encompasses the transition of $[S_0]$ going from being most limiting to being nonlimiting to the reaction. At $[S_0]$ exclusively $< K_m$ or $> K_m$, there is bias in the data set (Fig. 14.7) toward either of the two linear portions of this plot, with few measurements corresponding to the zone of curvature in (Fig. 14.7 inset).

Obtaining accurate measurements of K_m is important because K_m provides a quantitative measure of enzyme–substrate complementarity in binding (when $K_m \approx K_s$), and such values can be used to compare relative affinities of competing substrates. Second, the combined determination of V_{max} ($\propto k_{cat}$) and K_m for competing substrates provides for a quantitative comparison of specificity (selectivity) of the enzyme among substrates through the use of the specificity constant, or V_{max}/K_m [Eq. (14.4)] (Fersht, 1985).

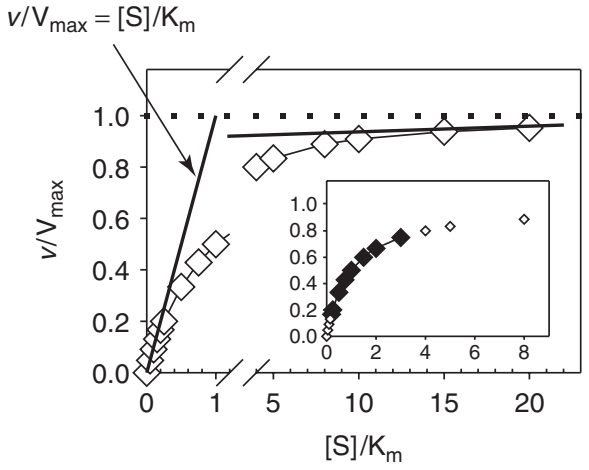

Figure 14.7. Conventional velocity (as a fraction of V_{max}) versus [S] (as a multiple of K_m) plot showing the two linear portions of a hyperbolic curve. Inset shows range of $[S]/K_m$ (♦) conducive to providing reliable estimates of V_{max} and K_m.

Studies that seek to compare specificity constants among different substrates under a defined set of conditions are often focused on the nature of enzyme–substrate interaction or structure–function relationships that confer reaction selectivity. In other cases, the determination of specificity constants for a single substrate under a variety of conditions is often an attempt to infer something about factors that govern or modulate reaction selectivity. In both cases, obtaining reliable data and estimates of kinetic constants are of paramount importance. The collection of observations in Table 14.1 provides an example of such a study, where different substrates were assayed over different ranges of [S] at a known [E] to yield estimates of k_{cat} and K_m.

The conclusions to be drawn for this type of study are likely to focus on the relationship between systematic changes in structural features of the substrates and the attendant changes in reaction selectivity (relative k_{cat}/K_m values). This may allow certain inferences to be drawn about the chemical nature of enzyme–substrate interactions that lead to productive binding and/or transition-state stabilization.

For example, a possible conclusion to be reached from the data in Table 14.1 is: "Reaction selectivity with substrate 7 was two orders of magnitude greater than for substrates 5 or 6". Based on structural differences between substrate 7, and 5 and 6, conclusions may be further delineated to suggest that specific functional groups of the substrate (and enzyme) may participate in catalysis by facilitating substrate binding or substrate transformation. Such conclusions would be valid or at least firmly supported if measurements of k_{cat} and K_m are accurate and reliable (Table 14.1).

It is a rather simple task to judge the reliability of this data set by calculating the K_m value (from the fourth and fifth columns in Table 14.1) and comparing it to the range of [S] values used (the second column in

TABLE 14.1 Selectivity Constants Determined for a Series of Substrates

Substrate (S)	Range of [S] Tested (mM)	Number of [S] Tested	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
1	0.50–2.5	6	0.897	296
2	1.0–6.0	8	0.184	36.0
3	0.50–8.0	6	2.97	1830
4	0.50–2.5	7	0.407	152
5	2.5–12.0	10	0.183	23.8
6	0.50–2.5	5	0.138	29.1
7	1.5–5.0	7	1.68	2260

TABLE 14.2 Assessment of Bias in [S] Range Used for Determining Selectivity Constants

Substrate (S)	Range of [S] Tested (mM)	Calculated K_m (mM)	Any Bias in [S]/K_m?
1	0.50–2.5	3.0	[S] $< K_m$
2	1.0–6.0	5.1	[S] $\leq K_m$
3	0.50–8.0	1.6	None
4	0.50–2.5	2.7	[S] $\leq K_m$
5	2.5–12.0	7.7	None
6	0.50–2.5	4.7	[S] $< K_m$
7	1.5–5.0	0.74	[S] $> K_m$

Table 14.1) for each substrate evaluated. This analysis is quite revealing in that the data set is biased for five of the seven substrates examined, such that estimates of both K_m and k_{cat} ($\propto V_{\max}$) may be quite erroneous (Table 14.2).

The scenario described above pertains to the design of experiments and collection of observations for the purpose of estimating $V_{\max}/K_m$ using conventional linear or nonlinear transformations. It should be pointed out that there is another approach to the measurement of $V_{\max}/K_m$, based on the principle that at low [S], the reaction velocity is proportional to $V_{\max}/K_m$ (Fig. 14.7). $V_{\max}/K_m$ approximates an apparent second-order rate constant (k_{cat}/K_m) describing the behavior of the free enzyme, but this relationship also holds at any [S] (Fersht, 1985). The utility of this relationship is founded on the fact that the relative velocities (v) of reactions between competing substrates is described as

$$\frac{v_{\text{A}}}{v_{\text{B}}} = \frac{(V_{\max}/K_m)_{\text{A}}[\text{S}]_{\text{A}}}{(V_{\max}/K_m)_{\text{B}}[\text{S}]_{\text{B}}} \tag{14.7}$$

From a practical point, each of several competing substrates may be incorporated into a reaction mixture at a single [S_0] value (they can be the same or different [S_0] values), and reactions may be allowed to proceed beyond the period where linear rates exist. Linear (log-log) transformations (Deleuze et al., 1987) are based on Eq. (14.7) and the relationships of

$$\frac{v_{\text{A}}}{v_{\text{B}}} = \alpha\frac{[\text{S}]_{\text{A}}}{[\text{S}]_{\text{B}}} \qquad \text{where} \qquad \alpha = \frac{(V_{\max}/K_m)_{\text{A}}}{(V_{\max}/K_m)_{\text{B}}} \tag{14.8}$$

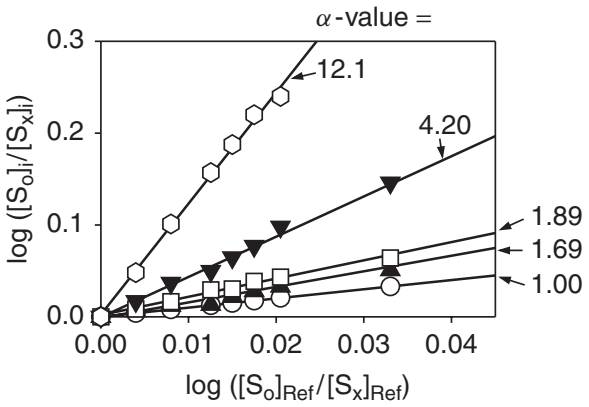

Figure 14.8. Log-log plots of enzyme reaction progress curves to provide estimates of relative $V_{\max}/K_m$ values (specificity constants). Different symbols are different substrates.

and

$$\log \frac{([S_0]_i)}{([S_x]_i)} = \alpha \log \frac{([S_0]_{\text{ref}})}{([S_x]_{\text{ref}})} \tag{14.9}$$

where $[S_0]$ and $[S_x]$ are the concentrations of substrate initially and at any time (respectively) during the reaction for any substrate (i) relative to a reference (ref) substrate. The log-log plots (Fig. 14.8) represent the fractional conversion of each substrate relative to $[S]_{\text{ref}}$ at all time intervals assayed. The ratios of the slopes of the linear plots are equivalent to the α values for the multiple comparisons that can be made.

Data used to construct these plots are useful to the point where there is a departure from linearity (usually, a downward deflection). The most likely causes for this departure from linearity include product inhibition, approaching reaction equilibrium, and enzyme inactivation during the course of reaction. These α values are *relative* quantities. However, if the actual $V_{\max}$ (or k_{cat}) and K_m values are determined accurately for one substrate (probably the reference), reasonable quantitative estimates of selectivity constants ($V_{\max}/K_m$) may be calculated for all the substrates in the series evaluated.

14.5 IS THERE CONSISTENCY WORKING WITHIN THE CONTEXT OF A KINETIC MODEL?

In this final section we examine a set of observations that may be interpreted in alternative ways: the point being that interpretation should be made within the context of any model that is evoked to represent enzyme kinetic behavior. The simplest and most commonly applied model, the

Michaelis–Menten or hyperbolic kinetics model, is used here to illustrate how a model can be employed to guide interpretations and conclusions.

Substrate inhibition in studies on enzyme kinetics is a property observed more often than perhaps one would anticipate. An example of an enzyme reaction subject to substrate inhibition is illustrated in Fig. 14.9. A conclusion that may be reached upon the presentation of such data is "... the enzyme reaction was subject to substrate inhibition at [S] of greater than 2 m*M*." This would be a naïve comment; a more a precise comment would be that "... the enzyme reaction was subject to substrate inhibition *and reaction rates started to decline* at [S] of greater than 2 m*M*." The difference between these statements lies much deeper than simply semantics.

To make an appropriate assessment of the pattern of inhibition, one need only compare the pattern of reaction velocity versus [S] observed relative to the pattern predicted from an application of the hyperbolic kinetics model. This requires making an estimate of $V_{\max}$ and K_m from the data available. Transforming the original data to a Lineweaver–Burke plot *(despite the aforementioned limitations)* indicates that only four data points (at low [S]) can be used to estimate $V_{\max}$ and K_m (as 3.58 units and 0.48 m*M*, respectively, Fig. 14.10). The predicted (uninhibited) behavior of the enzyme activity can now be calculated by applying the rectangular hyperbola [Eq. (14.5)] (yielding the upper curve in Fig. 14.11), and it becomes clear that inhibition was obvious at [S] $\leq$1 m*M*. The degree of inhibition is expressed appropriately as the difference between observed and predicted activity at any [S] value, if one makes interpretations within the context of the Michaelis–Menten model.

Because of the leveling off of enzyme activity at 3 to 5 m*M* [S] (Fig. 14.9), another conclusion that may be reached through intuition is that "... this pattern of activity can be explained by the presence of two

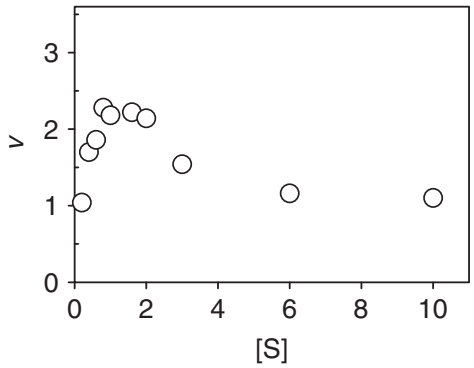

Figure 14.9. Rate data for an enzyme subject to substrate inhibition.

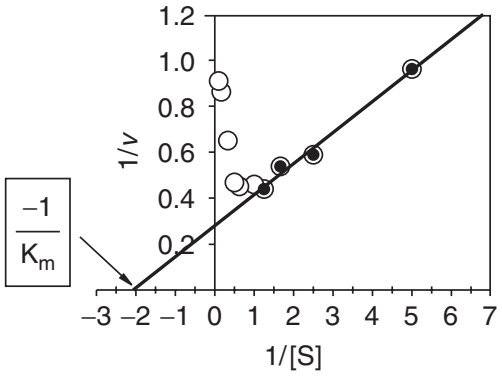

Figure 14.10. Data from Fig. 14.9 transformed to a double-reciprocal plot. Only some data (◉) were used to construct the linear plot and allow estimates of V_{max} and K_m.

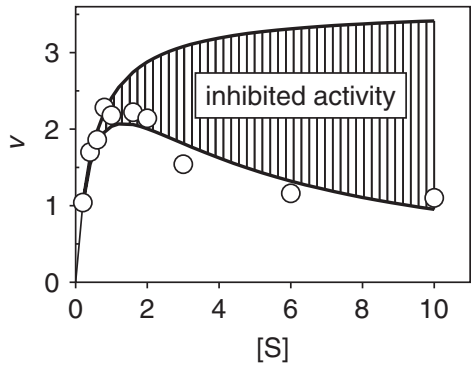

Figure 14.11. Example rate data in Fig. 14.9 (○) contrasted with the predicted behavior (upper curve) of an uninhibited enzyme with the V_{max} and K_m values derived from Fig. 14.10.

enzymes that act on this substrate, one enzyme subject to substrate inhibition, and the other enzyme not subject to substrate inhibition." To assess this statement, one must attempt to account mechanistically for the nature of enzyme inhibition by substrate. One can envision the nature of substrate inhibition using a modified form of the model in Eq. (14.1):

$$\begin{array}{l} \mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \\ +2\ \mathrm{S} \\ \Big\Vert\!\!\downarrow K_\mathrm{I} \\ \mathrm{ESS} \end{array} \tag{14.10}$$

where the added feature is the process whereby two molecules of S bind at the active site to form a deadend (nonproductive) complex, characterized

by a dissociation constant (K_I) for the inhibited enzyme species (ESS):

$$K_I = \frac{[E][S]^2}{[ESS]} \tag{14.11}$$

Conceptually, this mode of inhibition can be visualized as each of two substrate molecules binding to different subsites of the enzyme active site, resulting in nonalignment of reactive groups (designated as "$*$") on E and S (Fig. 14.12). Using the conventional approach of deriving the reaction velocity expressions yields

$$v = \frac{V_{max}[S]}{K_m + [S] + (K_m[S]^2)/K_I} \tag{14.12}$$

This relationship takes the form of the original rectangular hyperbola [Eq. (14.5)] modified by the incorporation of the substrate inhibition step:

$$y = \frac{ax}{b + x + bx^2/c} \tag{14.13}$$

Since a and b were determined earlier (Fig. 14.10), the equation only needs to be solved for c (K_I). There are at least two ways to solve for

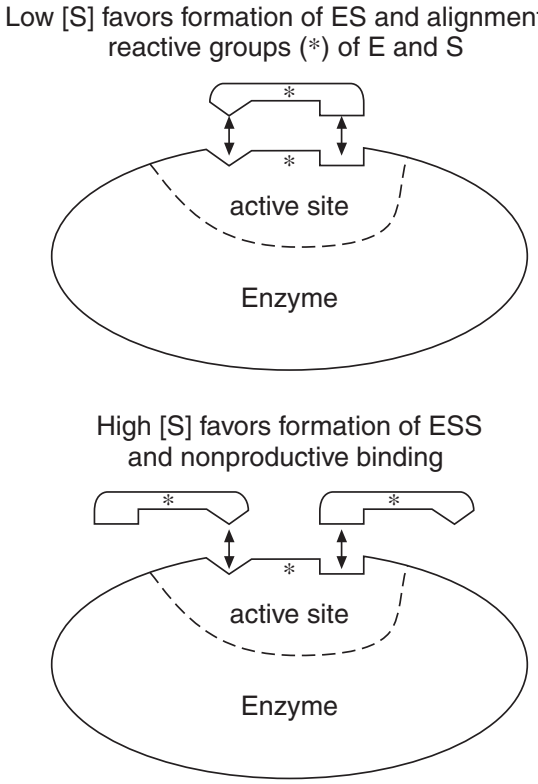

Figure 14.12. Visualization of model derived for substrate inhibition of enzyme in Eq. (14.10).

K_I, one of which is through nonlinear regression fitting of the actual data using the relationship just described [Eq. (14.13)], and this yields a value for K_I of 1.85 mM ($r^2 = 0.98$). A second and nonconventional way is to use Fig. 14.10 and consider the points corresponding to the four greatest [S] as observations in the presence of competitive inhibitor (Fig. 14.13). This provides four estimates of K_I if the plot is interpreted as behaving by classical competitive inhibition kinetics (the exception being that the $[S]^2$ and not [I] parameter [based on scheme (14.10)] is used in the term corresponding to the x-intercept). The mean of these four estimates of K_I is 1.78 mM (with a narrow range of 1.2 to 2.2 mM), very close to the 1.85 mM value determined by nonlinear regression.

Based on the two analyses just described, a K_I value of 1.8 mM was used and the pattern of enzyme activity predicted using the model [Eqs. (14.10) through (14.13)] is shown as the lower curve in Fig. 14.11. It is apparent that although there is some systematic deviation of the actual data from the curve modeling substrate inhibition, the approximation to the data observed is nonetheless reasonable.

To further evaluate the alternative views of the presence of one versus two enzymes, one could proceed with evaluating how well the data fit a two-enzyme model. In this scenario one is forced to make certain assumptions about the relative kinetic properties and contribution of each enzyme to the behavior observed in Fig. 14.9. For the sake of this analysis, the

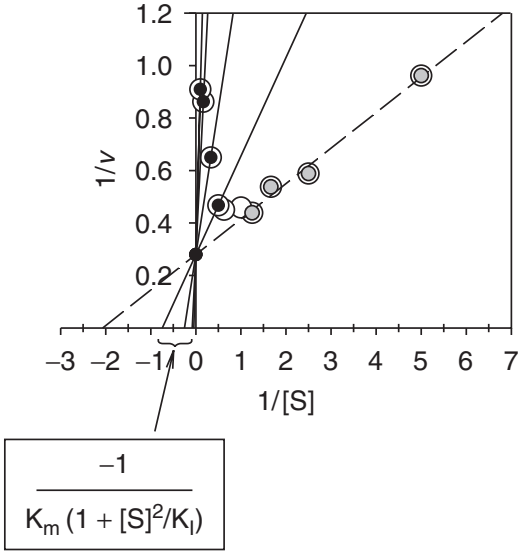

Figure 14.13. Same plot as Fig. 14.10 except for the addition of four plots at high [S] value (◉) modeled as competitive inhibition by substrate. Intersects at $1/V_{max}$ were constructed to arrive at four separate estimates of inhibition constant (K_I) based on the model in Eqs. (14.10) and (14.11). Original estimates of K_m and V_{max} were based on the data used to construct the broken line plot, as in Fig. 14.10.

assumptions made here are that:

1. The K_m values for the two enzymes are the same (primarily because without any further information, it would be difficult to assume a priori that one enzyme has a greater or lesser K_m value than the other).
2. The relative contribution of activity of each enzyme at [S] = 10 mM is equal.

Based on these assumptions, the contribution of the second, noninhibited enzyme to the data observed (Fig. 14.9) can be calculated. The data observed can now be partitioned into the individual contributions of the two enzymes (Fig. 14.14*a*). The lower curve represents the uninhibited enzyme and the upper curve represents the inhibited enzyme, which is

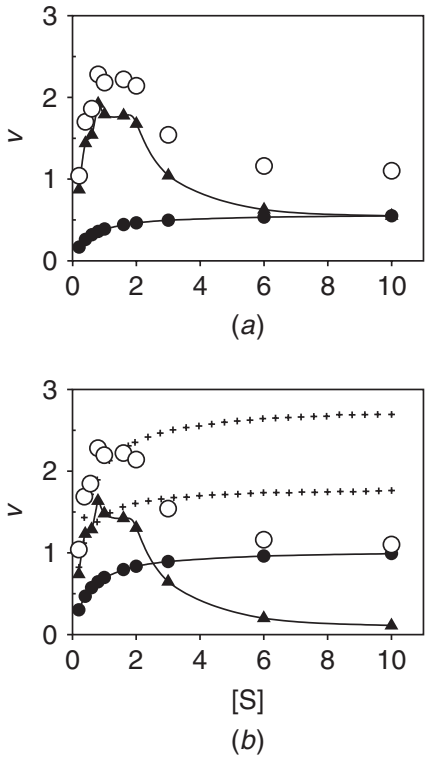

Figure 14.14. Modeling of a two-enzyme system, with one enzyme subject to substrate inhibition (▲) and the other not inhibited by substrate (●) using the data in Fig. 14.9 (○). (*a*) Both enzymes are assumed to have the same K_m and make equal contributions to activity observed at 10 mM [S]. (*b*) Both enzymes are assumed to have the same K_m and the uninhibited enzyme contributes 90% of the activity observed at 10 mM [S]. Additional plots (+ + +) in (*b*) predict the behavior of an enzyme subject to substrate inhibition by binding only one molecule to S to form an inactive E′S complex with a K_I value of 1.8 mM (upper curve) or 0.5 mM (lower curve).

calculated as the difference between the data observed (open symbols) and the contribution of the uninhibited enzyme.

One now needs to evaluate how well the inhibition constant (K_I) can afford a fit to the pattern predicted for the inhibited enzyme (upper curve in Fig. 14.14*a*) of the two-enzyme model. One approach would be to apply a nonlinear regression (which in this case did not allow for convergence or a good fit). An alternative approach is a more pencil-and-paper type of exercise to test the inhibited enzyme (of the two-enzyme model) for fit by rearranging Eq. (14.12) to solve for K_I by calculating K_I for the inhibited enzyme component for each datum point or observation made:

$$K_I = \frac{K_m/[S]}{(V_{max}/v) - (K_m/[S]) - 1} \tag{14.14}$$

This was done first for the original data (Fig. 14.9) after estimating K_m and V_{max} (Fig. 14.10) and omitting the first four observations at [S] $\leq$ 4 mM because some "nonsense" or negative numbers were obtained (the extent of inhibition at low [S] is negligible and may be difficult to decipher). The single-enzyme system subject to substrate inhibition and modeled by the lower curve in Fig. 14.11 had a calculated [using Eq. (14.14)] mean K_I value of 2.2 mM (range 1.3 to 3.2 mM, again very close to the 1.8 mM value derived from the two other approaches employed). When these same data are modeled as a two-enzyme system, the inhibited enzyme was characterized by a calculated [using Eq. (14.14)] K_I value of 1.5 mM (range 0.79 to 2.6 mM). This analysis and the calculation of mean (and range of) K_I provide little as a basis to differentiate conclusively between the ability of one model to fit the observations better than the other, and in this case, the most conservative approach would be to conclude that the simpler (one-enzyme) model is valid.

Furthermore, if one modifies the assumptions to have the noninhibited enzyme in the two-enzyme model constitute a greater proportion (e.g., about 90%) of the activity observed at the greatest [S] (10 mM) (Fig. 14.14*b*), the calculation of K_I [using Eq. (14.14)] is subject to less precision (mean of 1.0 mM and range of 0.22 to 2.2 mM), and there is a systematic decline in K_I as one progresses toward greater [S]. Thus, the more the two-enzyme system model is emphasized in the analysis, the less it fits the observed data, whereas a single-enzyme system (Fig. 14.11) appears to explain the observations sufficiently well.

Finally, a model for substrate inhibition alternative to Eqs. (14.10) and (14.11) was evaluated by testing if a nonproductive E–S complex could involve only one (and not two) molecules of bound substrate (E′S as the inhibited species as opposed to ESS). This was done using the

kinetic constants (V_{max} and K_m) derived earlier from Fig. 14.10 and K_I values of 1.8 and 0.5 mM. The resulting plot predicted by this alternative model are the two curves indicated by plus signs (+) for these respective K_I values in Fig. 14.14(*b*). It is obvious that simple enzyme inhibition by a single molecule of bound substrate does not predict the cooperative inhibitory effect of high [S] (2 to 10 mM in Fig. 14.9) as well as does the model depicted in Eq. (14.10).

14.6 CONCLUSIONS

The purpose of this chapter is to illustrate how the application of simple kinetic principles and relationships are critical to analyzing and reaching appropriate conclusions for experimental observations on enzyme kinetic properties. Many misrepresentations or errors in interpretation of experimental data can be avoided by working within (or verifying the applicability of) a kinetic model and not relying on intuition. Resisting the immediate temptation to linearize the original data and analyze the transformed data without careful consideration would also help!

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