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SIMPLIFIED VELOCITY EQUATIONS FOR CHARACTERIZING THE PARTIAL INHIBITION OR NONESSENTIAL ACTIVATION OF BIREACTANT ENZYMES

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The steady state velocity equation for a bireactant enzyme in the presence of a partial inhibitor or nonessential activator, M, contains squared substrate concentration and higher-ordered M concentration terms. The equation is too complex to be useful in kinetic analyses. Simplification by the method of Cha (*J. Biol. Chem.* **243**, 820–825 (1968)) eliminates squared substrate concentration terms, but retains higher-ordered terms in [M]. It is shown that if strict equilibrium is assumed between free E, M, and EM and for all but one other M-binding reaction, a velocity equation is obtained for an ordered bireactant enzyme that is first degree in all ligands in the absence of products. The equation is an approximation (because it was derived assuming only one M-binding reaction in the steady state), but it contains five inhibition (or activation) constants associated with M, all of which can be obtained by diagnostic replots and/or curve-fitting procedures. The equation also provides a framework for obtaining limiting constants (V'_{max}, K'_{ia}, K'_{mA}, K'_{mB}) that characterize the enzyme at saturating M. The same approach is applicable to an enzyme that catalyzes a steady state ping pong reaction.

Keywords: Inhibition, partial; Activation, nonessential; Bireactant mechanisms, partial inhibition in; Kinetics of partial systems; Hyperbolic effectors; Modifier, general mechanism kinetics

INTRODUCTION

Enzyme inhibitors, I, can be divided into two broad classes: those that drive the reaction rate toward zero as $[I] \rightarrow \infty$, and those that drive the rate

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toward some finite limit as $[I] \rightarrow \infty$. The former are called *complete* inhibitors and if only one molecule of inhibitor binds per molecule of enzyme (the classical case), the diagnostic slope and/or $1/v$-axis intercept replot of the primary double reciprocal plots will be linear. (The primary plots are $1/v$ versus $1/[S]$ at different fixed levels of I.) Consequently, such inhibitors are also called *linear* inhibitors. Inhibitors that drive the reaction to a finite limit are called *partial* or *hyperbolic* inhibitors, the term "hyperbolic" referring to the shape of the diagnostic replots. Activators can also be divided into two classes: *essential* (which are absolutely necessary for the reaction to proceed) and *nonessential*. An essential activator can usually be treated as if it were a cosubstrate. Nonessential activators are kinetically similar to partial inhibitors, except that the effects are in the opposite direction. (There is a finite velocity in the absence of the activator and the velocity rises to a finite higher limit as the activator $\rightarrow \infty$; slope and/or intercept replots are hyperbolic functions of the activator concentration.) Methods for analyzing the effects of linear inhibitors and essential activators on bireactant enzymes that follow steady state kinetics are well known.[1-3] However, investigators evaluating the kinetics of partial inhibition and nonessential activation usually resort to the assumption that the enzyme is unireactant.[4-12] As a result, the number of inhibition or activation constants that can be obtained is reduced and most of the experimental constants are only apparent ones which depend on an arbitrarily-chosen concentration of the cosubstrate. The reason for the assumption of unireactivity is that a steady state treatment of bireactant enzymes in the presence of a partial effector produces velocity equations that are too complex to be useful for either curve fitting or analyses by means of diagnostic plots. In this report, we suggest simplifying assumptions that yield useful equations for the partial effector ("general modifier") mechanism as applied to common steady state bisubstrate reactions.**

METHODS

Most mathematical manipulations were performed on a Power Macintosh 7500/100 or 8500/300. Simultaneous equations were solved with Mathcad

**This analysis does not include the kinetic effects of an alternative substrate which can behave as a partial effector when present together with the "normal" substrate if v is taken as the rate of common product formation.

PLUS, version 6. Microsoft Excel 4.0 was used for simulation calculations. Curve fits were performed using DeltaGraph 4.05c.

REACTION SCHEME AND STEADY STATE EQUATIONS

Ordered Bi Bi Mechanism

To illustrate the consequences of different simplifying assumptions, consider a steady state compulsory ordered bi bi sequence. The reactions that occur in the presence of a partial effector, M, that binds to all enzyme species are shown in Figure 1.

Clearly, M must bind at a site that does not include or overlap the catalytic site. The effect of M is to establish an alternative path for the binding of substrates A and B, catalysis, and the release of products P and Q. The parallel paths (shown horizontally) are linked by reversible M-binding reactions (shown vertically). Modifier M can act as a partial inhibitor or as a

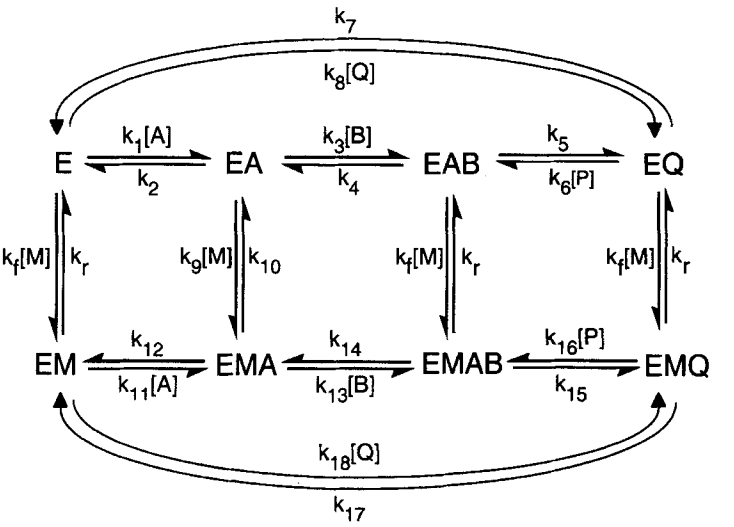

FIGURE 1 Reaction scheme for a steady state ordered bi bi reaction in the presence of an effector that binds to all enzyme species. The terms above and below the double arrows indicate the rate constant and ligand requirements for the reaction. For simplicity, the catalytic step EAB $\rightleftharpoons$ EPQ is not explicitly shown, i.e., EAB represents EAB + EPQ. This omission has no effect on the final velocity equation. (It just means that some rate constants are actually composite constants. For example, k_5 and k_{15} account for catalysis plus the release of P. Similarly, k_6 and k_{16} account for the addition of P plus catalysis in the reverse direction.) Some of the rate constants for M addition and dissociation are indicated simply as k_f and k_r.

nonessential activator depending on whether the M-dependent path is faster or slower than the path without M at some fixed [A] and [B]. Under steady state conditions, the concentrations of all enzyme species remain constant as substrates A and B are converted to products P and Q. The equations describing this situation in the absence of products are shown below. (The k_r and k_f terms are not numbered because they will not appear in the final equation.)

$$\frac{d[E]}{dt} = 0 \quad \therefore k_2[EA] + k_7[EQ] + k_r[EM] = k_f[E][M] + k_1[E][A] \tag{1}$$

$$\frac{d[EA]}{dt} = 0 \quad \therefore k_1[E][A] + k_4[EAB] + k_{10}[EMA] = k_9[EA][M] + k_3[EA][B] + k_2[EA] \tag{2}$$

$$\frac{d[EAB]}{dt} = 0 \quad \therefore k_3[EA][B] + k_r[EMAB] = k_f[EAB][M] + k_4[EAB] + k_5[EAB] \tag{3}$$

$$\frac{d[EQ]}{dt} = 0 \quad \therefore k_5[EAB] + k_r[EMQ] = k_f[EQ][M] + k_7[EQ] \tag{4}$$

$$\frac{d[EM]}{dt} = 0 \quad \therefore k_{12}[EMA] + k_{17}[EMQ] + k_f[E][M] = k_r[EM] + k_{11}[EM][A] \tag{5}$$

$$\frac{d[EMA]}{dt} = 0 \quad \therefore k_{11}[EM][A] + k_{14}[EMAB] + k_9[EA][M] = k_{10}[EMA] + k_{13}[EMA][B] + k_{12}[EMA] \tag{6}$$

$$\frac{d[EMAB]}{dt} = 0 \quad \therefore k_{13}[EMA][B] + k_f[EAB][M] = k_r[EMAB] + k_{15}[EMAB] + k_{14}[EMAB] \tag{7}$$

$$\frac{d[EMQ]}{dt} = 0 \quad \therefore k_{15}[EMAB] + k_f[EQ][M] = k_r[EMQ] + k_{17}[EMQ] \tag{8}$$

and

$$[E]_t = [E] + [EA] + [EAB] + [EQ] + [EM] + [EMA] + [EMAB] + [EMQ] \tag{9}$$

The above series of simultaneous equations can be solved for the concentrations of all enzyme species. The King–Altman method[3,13] yields [E], [EM], [EMA], etc. in terms of $[E]_t$. A computer-assisted solution may do the same or yield the concentrations of seven of the species in terms of the eighth. The steady state velocity is obtained by substituting the solutions into Equation (10):

$$v/[E]_t = \{(k_7[EQ] + k_{17}[EMQ]) / ([E] + [EA] + [EAB] + [EQ] + [EM] + [EMA] + [EMAB] + [EMQ])\} \quad (10)$$

The final equation for the forward reaction velocity contains $[A]^2$, $[B]^2$, $[A][B]^2$, and $[A]^2[B]$ terms along with $[M]^n$, $[A][M]^n$, $[B][M]^n$, $[A]^2[M]^n$, $[A]^2[B][M]^n$ (etc.) up to $n=4$. If the system is treated as bi uni with EAB producing E + P, the final equation will still contain M terms up to $[M]^3$. In either case the equation may be exact, but it is not useful for obtaining constants associated with effector M.

SIMPLIFICATION BY THE QUASI-EQUILIBRIUM METHOD OF CHA

The most common approach to dealing with steady state systems containing random sequences (the major cause of the higher powered terms) is to use the method of Cha[14] to derive the velocity equation. Cha's method assumes that one or more reactions within the overall random sequence is at equilibrium. The equilibrium segments are treated as separate entities which are linked by nonequilibrium reactions in the steady state. For the ordered sequence shown in Figure 1 with all M-binding reactions at equilibrium, E plus EM would constitute one equilibrium segment (which is denoted as "W"), EA plus EMA would constitute another equilibrium segment (denoted "X"), EAB plus EMAB the third equilibrium segment ("Y"), and EQ plus EMQ the fourth ("Z"). These segments are interconverted by the reactions shown in Figure 2.

Each k in Figure 2 is the rate constant for a specific reaction. The corresponding f factor represents the fraction of a given equilibrium segment that participates in that reaction. For example, k_1 is the rate constant for the reaction of A with E (of segment W) to form EA (of segment X). f_1 is the fraction of segment W represented by E. Constant k_{11} is the rate constant

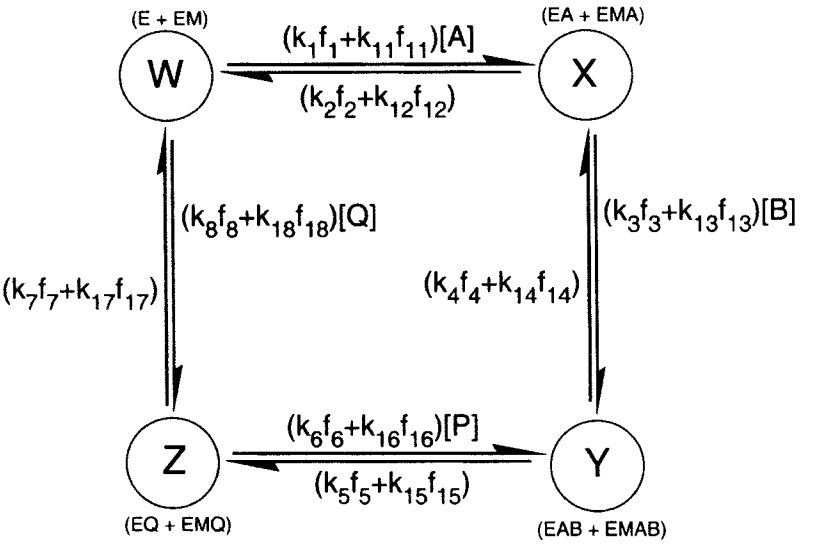

FIGURE 2 Interconversion of the equilibrium M-binding segments in the ordered bi bi sequence. The figure could be used as the basic King–Altman figure for determining the concentrations of enzyme species.

for the reaction of EM with A to form EMA; f_{11} is the fraction of segment W represented by EM. Similarly, k_2 and f_2 are, respectively, the rate constant for the dissociation of EA and the fraction of segment X represented by EA, while the terms k_{12} and f_{12} describe the dissociation of A from EMA. The fractional concentration factors can be obtained from equilibrium considerations, as shown below for f_2 and f_{12} where $K_d = k_{10}/k_9$.

$$f_2 = \frac{[EA]}{[X]} = \frac{[EA]}{[EA] + [EMA]} = \frac{1}{(1 + [M]/K_d)} = \frac{k_{10}}{k_{10} + k_9[M]} \qquad (11)$$

and

$$f_{12} = \frac{[EMA]}{[X]} = \frac{[EMA]}{[EA] + [EMA]} = \frac{[M]/K_d}{(1 + [M]/K_d)} = \frac{k_9[M]}{k_{10} + k_9[M]} \qquad (12)$$

The other f factors have the same form. The steady state equations for the various segments are written in the usual way, e.g.,

$$\frac{d[X]}{dt} = 0 \quad \therefore (k_1 f_1 + k_{11} f_{11})[W][A] + (k_4 f_4 + k_{14} f_{14})[Y]$$
$$= (k_2 f_2 + k_{12} f_{12})[X] + (k_3 f_3 + k_{13} f_{13})[X][B] \qquad (13)$$

Solving the series of steady state equations for W, X, Y, and Z in terms of $[E]_t$ (or solving for W, X, and Y in terms of Z) and substituting the results and the *f* factors into Equation (14) –

$$\frac{v}{[E]_t} = \frac{(k_7 f_7 + k_{17} f_{17})[Z]}{[W] + [X] + [Y] + [Z]} \tag{14}$$

yields a velocity equation that is first degree in [A] and [B] in the absence of products, but still contains various $[A][M]^n$, $[B][M]^n$, and $[A][B][M]^n$ terms with $n = 1$–4. The final equation contains 20 constants associated with M (although not all of them may be independent).

SIMPLIFICATION BY THE STRICT EQUILIBRIUM METHOD

If there is no indication that higher power [M] terms must be considered (e.g., the double reciprocal plots in the presence of M appear to be linear with hyperbolic slope and intercept replots), then the alternative approach suggested by Segel and Martin[15] can be used to obtain an equation that is first degree in substrate and modifier concentration. This method, like that of Cha, assumes equilibrium between certain species, but the equilibrium assumption is used differently, viz. to eliminate terms from the steady state equations for various enzyme species. That is, if the reaction $E + M \leftrightharpoons EM$ is at equilibrium, then $k_f[E][M]$ must equal $k_r[EM]$ and consequently, these two terms will cancel each other when they appear on opposite sides of an equation. In order to obtain a first degree equation for the ordered bireactant mechanism shown in Figure 1, we must assume that the binding of M to free E and to any two other species are at equilibrium. We will assume that the other two equilibrium reactions are $EAB + M \leftrightharpoons EMAB$ and $EQ + M \leftrightharpoons EMQ$. This eliminates all k_f- and k_r-containing terms except for those in the equations for $d[EA]/dt = 0$ and $d[EMA]/dt = 0$. The resulting steady state equations are the same as if it were assumed that M bound initially only to EA (and then distributed into EMAB, EMQ, and EM via the reactions of the lower pathway shown in Figure 1).

Solving the modified Equations (1)–(8) for each species† and then substituting the results into Equation (10) yields (after grouping similar terms)

† If the method of King and Altman[3,13] is used, the terms for all species will contain either an [A] or an $[A]^2$ factor when $[P] = 0$ and $[Q] = 0$. Consequently, an [A] can be cancelled from all terms before or after substitution into Equation (10).

Equation (15)

$$v = \{(\text{num}_1[A][B][E]_t + \text{num}_2[A][B][M][E]_t) / (\text{Const} + \text{Coef}_A[A] + \text{Coef}_B[B] + \text{Coef}_{AB}[A][B] + \text{Coef}_M[M] + \text{Coef}_{AM}[A][M] + \text{Coef}_{BM}[B][M] + \text{Coef}_{ABM}[A][B][M])\} \quad (15)$$

Defining ratios of coefficients as prescribed by Cleland[1,2] we obtain a final velocity equation that is first degree in all ligands

$$v = \{(V_{max}[A][B](1 + [M]/K_5)) / (K_{ia}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B] + (K_{ia}K_{mB}[M])/K_1 + (K_{mB}[A][M])/K_2 + (K_{mA}[B][M])/K_3 + ([A][B][M])/K_4)\} \quad (16)$$

The above method does not simply yield the rapid equilibrium counterpart of the steady state mechanism. That is, Equation (16) is *not* the same as that for a rapid equilibrium ordered mechanism in the presence of a partial effector. (That equation would not contain [B] and [B][M] denominator terms.) Actually, Equation (16) has the same form as that for a rapid equilibrium *random* mechanism in which M binds to all species (see Segel[3] pp. 293–295). This identity was not unexpected because (a) the velocity equations for the steady state ordered and rapid equilibrium random mechanisms are identical in the absence of M and (b) the presence of M does not change the binding order of substrates A and B.

The limiting kinetic constants in the absence of M are defined as follows:

$$\frac{V_{max}}{[E]_t} = \frac{\text{num}_1}{\text{Coef}_{AB}} = \frac{k_5k_7}{(k_5 + k_7)},$$

$$K_{ia} = \frac{\text{Const}}{\text{Coef}_A} = \frac{k_2}{k_1},$$

$$K_{mA} = \frac{\text{Coef}_B}{\text{Coef}_{AB}} = \frac{k_5k_7}{k_1(k_5 + k_7)},$$

$$K_{mB} = \frac{\text{Coef}_A}{\text{Coef}_{AB}} = \frac{k_7(k_4 + k_5)}{k_3(k_5 + k_7)},$$

$$K_1 = \frac{\text{Const}}{\text{Coef}_M} = \frac{k_2k_{10}k_{11}}{k_1k_9k_{12}},$$

$$K_2 = \frac{\text{Coef}_A}{\text{Coef}_{AM}} = \frac{k_{10}}{k_9},$$

$$K_3 = \frac{\text{Coef}_B}{\text{Coef}_{BM}} = \frac{(k_{14}+k_{15})k_3k_5k_{10}k_{11}}{(k_4+k_5)k_1k_9k_{13}k_{15}},$$

$$K_4 = \frac{\text{Coef}_{AB}}{\text{Coef}_{ABM}} = \frac{(k_5+k_7)(k_{14}+k_{15})k_3k_{10}k_{17}}{(k_4+k_5)(k_{15}+k_{17})k_7k_9k_{13}},$$

and

$$K_5 = \frac{\text{num}_1}{\text{num}_2} = \frac{(k_{14}+k_{15})(k_3k_5k_{10})}{(k_4+k_5)(k_9k_{13}k_{15})}.$$

Note that K_2 is equivalent to the M dissociation constant of the EMA complex. The Principle of Microscopic Reversibility dictates that the product of the rate constants in one direction around a series of reactions in a loop equals the product of the rate constants in the opposite direction. Consequently, there may be alternative ways of defining some of the above kinetic constants. For example, around the left-most square of Figure 1, $k_1k_9k_{12}k_r = k_fk_{11}k_{10}k_2$. Thus, $K_1 = k_2k_{10}k_{11}/k_1k_9k_{12} = k_r/k_f =$ the dissociation constant of EM.

The limiting constants V_{max}, K_{ia}, K_{mA}, and K_{mB} are determined from initial velocity measurements made in the absence of M. We can also define limiting constants that describe the mechanism at saturating [M]:

$$\frac{V'_{max}}{[E]_t} = \frac{\text{num}_2}{\text{Coef}_{ABM}} = \frac{k_{15}k_{17}}{(k_{15}+k_{17})},$$

$$K'_{ia} = \frac{\text{Coef}_M}{\text{Coef}_{AM}} = \frac{k_{12}}{k_{11}},$$

$$K'_{mA} = \frac{\text{Coef}_{BM}}{\text{Coef}_{ABM}} = \frac{k_{15}k_{17}}{k_{11}(k_{15}+k_{17})},$$

$$K'_{mB} = \frac{\text{Coef}_{AM}}{\text{Coef}_{ABM}} = \frac{k_{17}(k_{14}+k_{15})}{k_{13}(k_{15}+k_{17})}$$

The rate constant compositions of the limiting kinetic constants (at zero and saturating [M]) are unaffected by the choice of the M-binding reaction included in the steady state. However, the rate constant compositions of K_1 through K_5 depend on the specific assumptions made. For example, if the EAB + M $\rightleftharpoons$ EMAB reaction were the one included in the steady state, then none of the K values associated with M would be a simple dissociation constant. Nevertheless, there would still be five constants associated with M

and the relationships between the constants would be the same, as shown below:

$$\frac{K_4}{K_5} = \frac{V'_{max}}{V_{max}}, \quad \frac{K_2}{K_1} = \frac{K'_{ia}}{K_{ia}},$$
$$\frac{K_4}{K_3} = \frac{K'_{mA}}{K_{mA}}, \quad \frac{K_4}{K_2} = \frac{K'_{mB}}{K_{mB}},$$

and also

$$\frac{K_3}{K_5} = \frac{k_{11}}{k_1}$$

When A is the varied substrate, the velocity equation can be written as

$$v = \frac{V_{max}[A]\left(1 + \frac{[M]}{K_5}\right)}{K_{mA}\left[1 + \frac{K_{ia}K_{mB}}{K_{mA}[B]}\left(1 + \frac{[M]}{K_1}\right) + \frac{[M]}{K_3}\right] + [A]\left[1 + \frac{K_{mB}}{[B]}\left(1 + \frac{[M]}{K_2}\right) + \frac{[M]}{K_4}\right]} \quad (17)$$

The $1/v$-axis intercept and slope of the $1/v$ versus $1/[A]$ plot at different fixed concentrations of M and a constant subsaturating level of B are hyperbolic functions of [M]:

$$\text{intercept} = \frac{1}{V_{max,app}} = \frac{[1 + (K_{mB}/[B])(1 + [M]/K_2) + [M]/K_4]}{V_{max}(1 + [M]/K_5)} \quad (18)$$

$$\text{slope} = \frac{K_{mA,app}}{V_{max,app}} = \frac{K_{mA}\left[1 + \frac{K_{ia}K_{mB}}{K_{mA}[B]}\left(1 + \frac{[M]}{K_1}\right) + \frac{[M]}{K_3}\right]}{V_{max}\left(1 + \frac{[M]}{K_5}\right)} \quad (19)$$

When B is the varied substrate:

$$v = \{(V_{max}[B](1 + [M]/K_5))$$
$$/(K_{mB}[1 + (K_{ia}/[A])(1 + [M]/K_1) + [M]/K_2]$$
$$+ [B][1 + (K_{mA}/[A])(1 + [M]/K_3) + [M]/K_4])\} \quad (20)$$

$$\text{intercept} = \frac{1}{V_{max,app}} = \frac{[1 + (K_{mA}/[A])(1 + [M]/K_3) + [M]/K_4]}{V_{max}(1 + [M]/K_5)} \quad (21)$$

$$\text{slope} = \frac{K_{mB,app}}{V_{max,app}} = \frac{K_{mB}[1 + (K_{ia}/[A])(1 + [M]/K_1) + [M]/K_2]}{V_{max}(1 + [M]/K_5)} \quad (22)$$

If the basic constants for substrates A and B are known, all of the kinetic constants associated with M can be obtained by a conventional analysis based on primary linear plots and secondary and tertiary replots. (A substantial number of measurements are necessary, but no more than that required to characterize any mechanism involving three ligands.) First, one would obtain several sets of $1/v$ versus $1/[A]$ and $1/v$ versus $1/[B]$ plots, each set composed of the plots at three or four different fixed concentrations of M and a constant subsaturating concentration of cosubstrate. Three or four more such sets of primary plots, each obtained at a different concentration of the "constant" cosubstrate, are also needed. The $1/v$-axis intercepts and slopes of the double reciprocal plots in each set are then replotted as $1/\Delta$intercept versus $1/[M]$ and $1/\Delta$slope versus $1/[M]$. If M is a typical partial inhibitor, Δ is taken as intercept (or slope) in the presence of M minus intercept (or slope) in the absence M. If M is a typical nonessential activator, the opposite difference is taken. Each of the $1/\Delta$ replots yields $-1/K_5$ as the horizontal axis intercept. The inverse of the vertical axis intercepts of these replots is then plotted against the reciprocal of the "constant" cosubstrate concentration. For example, consider a family of $1/v$ versus $1/[A]$ plots at different fixed [M] and a single constant [B]. The secondary $1/\Delta$intercept replot is given by

$$\frac{1}{\Delta\text{int}} = \frac{V_{\max}K_2K_4K_5[B]}{K_{mB}K_4(K_5 - K_2) + K_2(K_5 - K_4)[B]}\frac{1}{[M]} + \frac{V_{\max}K_2K_4[B]}{K_{mB}K_4(K_5 - K_2) + K_2(K_5 - K_4)[B]} \tag{23}$$

When $1/\Delta\text{int} = 0$, $1/[M] = -1/K_5$. The vertical-axis intercept is equivalent to $1/\Delta$int at saturating M and the subsaturating B. As there will be secondary $1/\Delta$int versus $1/[M]$ replots for several other "constant" [B], the reciprocal of the vertical-axis intercepts (denoted $\Delta\text{int}_{\max}$ can be replotted against $1/[B]$:

$$\Delta\text{int}_{\max} = \frac{K_{mB}}{V_{\max}}\left(\frac{K_5}{K_2} - 1\right)\frac{1}{[B]} + \frac{1}{V_{\max}}\left(\frac{K_5}{K_4} - 1\right) \tag{24}$$

With K_5 known, K_2 and K_4 can be calculated from the slope and vertical-axis intercept, respectively, of this tertiary replot.

The $1/\Delta$slope replots are given by

$$\frac{1}{\Delta\text{slope}} = \frac{V_{\max}K_1K_3K_5[\text{B}]}{K_{\text{ia}}K_{\text{mB}}K_3(K_5-K_1)+K_{\text{mA}}K_1(K_5-K_3)[\text{B}]}\frac{1}{[\text{M}]} + \frac{V_{\max}K_1K_3[\text{B}]}{K_{\text{ia}}K_{\text{mB}}K_3(K_5-K_1)+K_{\text{mA}}K_1(K_5-K_3)[\text{B}]} \tag{25}$$

This secondary replot also yields $-1/K_5$ as the horizontal-axis intercept. Then, the tertiary $\Delta\text{slope}_{\max}$ versus $1/[\text{B}]$ replot yields K_1 and K_3:

$$\Delta\text{slope}_{\max} = \frac{K_{\text{ia}}K_{\text{mB}}}{V_{\max}}\left(\frac{K_5}{K_1}-1\right)\frac{1}{[\text{B}]}+\frac{K_{\text{mA}}}{V_{\max}}\left(\frac{K_5}{K_3}-1\right) \tag{26}$$

With K_{ia}, K_{mA}, $V_{\max}$, etc. and K_1 through K_5 known, all the limiting constants for saturating M can be calculated.

Starting instead with families of $1/v$ versus $1/[\text{B}]$ plots, the above replotting procedure yields the same inhibition constants. The relevant equations are:

$$\frac{1}{\Delta\text{int}} = \frac{V_{\max}K_3K_4K_5[\text{A}]}{K_{\text{mA}}K_4(K_5-K_3)+K_3(K_5-K_4)[\text{A}]}\frac{1}{[\text{M}]} + \frac{V_{\max}K_3K_4[\text{A}]}{K_{\text{mA}}K_4(K_5-K_3)+K_3(K_5-K_4)[\text{A}]} \tag{27}$$

$$\Delta\text{int}_{\max} = \frac{K_{\text{mA}}}{V_{\max}}\left(\frac{K_5}{K_3}-1\right)\frac{1}{[\text{A}]}+\frac{1}{V_{\max}}\left(\frac{K_5}{K_4}-1\right) \tag{28}$$

$$\frac{1}{\Delta\text{slope}} = \frac{V_{\max}K_1K_2K_5[\text{A}]}{K_{\text{ia}}K_{\text{mB}}K_2(K_5-K_1)+K_{\text{mB}}K_1(K_5-K_2)[\text{A}]}\frac{1}{[\text{M}]} + \frac{V_{\max}K_1K_2[\text{A}]}{K_{\text{ia}}K_{\text{mB}}K_2(K_5-K_1)+K_{\text{mB}}K_1(K_5-K_2)[\text{A}]} \tag{29}$$

and

$$\Delta\text{slope}_{\max} = \frac{K_{\text{ia}}K_{\text{mB}}}{V_{\max}}\left(\frac{K_5}{K_1}-1\right)\frac{1}{[\text{A}]}+\frac{K_{\text{mB}}}{V_{\max}}\left(\frac{K_5}{K_2}-1\right) \tag{30}$$

Some limiting constants can be obtained directly from the tertiary replots. For example, the vertical-axis intercept of Equation (24) is equivalent to $(1/V'_{\max})-(1/V_{\max})$, from which $V'_{\max}$ can be calculated. The intercept of

the corresponding tertiary Δslope_{max} replot (Equation (26)) is equivalent to $(K'_{mA}/V'_{max}) - (K_{mA}/V_{max})$. So with V_{max}, K_{mA}, and V'_{max} known, K'_{mA} can be determined. Similarly, the tertiary Δslope_{max} replot of the $1/v$ versus $1/[B]$ data (Equation (30)) will provide K'_{mB}.

Instead of (or in addition to) the linear replots described above, curve fits to the intercept and slope data can be used to obtain the apparent constants at saturating M for different fixed cosubstrate levels. For example, the intercept replot described by Equation (18) is a hyperbola with a nonzero origin (as shown later in Figure 6(b)). The numerical data can be fitted to general Equation (31):

$$\text{intercept} = \text{int}_{(-M)} + \frac{[M](\Delta\text{max})}{c + [M]} \tag{31}$$

where $\text{int}_{(-M)}$ is the known intercept at the fixed subsaturating B in the absence of M, Δmax is the maximum change in the intercept (which will be observed when $[M] = \infty$), and c is an apparent constant (equivalent to the [M] that yields half of the maximum intercept change). It can be shown that $c = K_S$. With Δmax so determined, the intercept at saturating M and the fixed subsaturating B can be calculated as $\text{int}_{(-M)} + \Delta\text{max}$. The $\text{int}_{@satM}$ is related to V'_{max} and K'_{mB} as shown by Equation (32)

$$\text{int}_{@satM} = \frac{(1 + K'_{mB}/[B])}{V'_{max}} \tag{32}$$

A similar curve fit to the slope versus [M] data again gives $c = K_S$ and allows the slope of the $1/v$ versus $1/[A]$ plot at saturating M and the subsaturating B to be determined. This value is related to limiting constants as shown by Equation (33)

$$\text{slope}_{@satM} = \frac{K'_{mA}}{V'_{max}}\left(1 + \frac{K'_{ia}K'_{mB}}{K'_{mA}[B]}\right) \tag{33}$$

The intercepts and slopes of the $1/v$ versus $1/[B]$ plots at saturating M and unsaturating A can be determined in the same way. These are related to limiting constants as shown below

$$\text{int}_{@satM} = \frac{(1 + K'_{mA}/[A])}{V'_{max}} \tag{34}$$

and

$$\text{slope}_{@\text{satM}} = \frac{K'_{\text{mB}}}{V'_{\text{max}}}\left(1 + \frac{K'_{\text{ia}}}{[\text{A}]}\right) \tag{35}$$

The intercepts and slopes at saturating M can then be replotted against the different "constant" 1/[A] or 1/[B] (or further curve-fitted) to yield K'_{mA}, K'_{ia}, K'_{mB}, and V'_{max}. With K_5 and the limiting constants at zero and saturating M known, K_1 through K_4 can be calculated.

Nonessential activation is analyzed in the same manner. The $1/\Delta$int and $1/\Delta$slope replots yield $-1/K_5$ as shown above. For the most common form of nonessential activation where the slopes and intercepts decrease with increasing [M], the relative positions of the constants in the difference terms of Equations (23) and (25) are reversed e.g., (K_2-K_5) instead of (K_5-K_2), (K_1-K_5) instead of (K_5-K_1), etc. Also, the difference terms of Equations (24) and (26) have the form $(1-K_5/K_2)$ and $(1-K_5/K_4)$. The equation analogous to Equation (31) would be intercept $=\text{int}_{(-\text{M})}$ – hyperbolic function. The limiting $V'_{\text{max,app}}$ would equal $\text{int}_{(-\text{M})}-\Delta\text{max}$.

If it is feasible to maintain each substrate at a level that is near saturating as the other is varied at different [M], then a shortcut may yield reasonable estimates of the constants for M. For example, from Equations (21) and (22) we see that at saturating [A], the intercept and slope of the $1/v$ versus 1/[B] plots reduce to:

$$\text{intercept} = \frac{(1 + [\text{M}]/K_4)}{V_{\text{max}}(1 + [\text{M}]/K_5)} \tag{36}$$

and

$$\text{slope} = \frac{K_{\text{mB}}(1 + [\text{M}]/K_2)}{V_{\text{max}}(1 + [\text{M}]/K_5)} \tag{37}$$

(To insure that the $[\text{M}]/K_1$ and $[\text{M}]/K_3$ terms remain insignificant, it may be advisable to increase [A] along with [M]. That is, have $[\text{A}] \gg K_{\text{mA}}$ and $\gg K_{\text{ia}}$ plus some constant multiple of [M].) Replots of $1/\Delta$int and $1/\Delta$slope versus 1/[M] or computer-assisted curve fitting of the intercepts and slopes to the above equations will yield K_2, K_4, and K_5. Similarly, replots or curve fitting to the intercept and slope of the $1/v$ versus 1/[A] plots at saturating [B] can yield K_3, K_4, and K_5. The remaining constant, K_1, can then be assessed from the value of a slope at subsaturating cosubstrate.

APPLICATION TO OTHER MECHANISMS

Ping Pong Bi Bi Mechanism

Figure 3 shows a ping pong mechanism in the presence of a partial inhibitor or nonessential activator that binds to all enzyme species. As before, the Cha method yields a final equation that is first degree in [A] and [B], but up to fourth degree in [M]. However, if it is assumed that any three of the four M-binding reactions are at strict equilibrium, we obtain:

$$v = \{(\mathrm{num}_1[A][B][E]_t + \mathrm{num}_2[A][B][M][E]_t) / (\mathrm{Coef}_A[A] + \mathrm{Coef}_B[B] + \mathrm{Coef}_{AB}[A][B] + \mathrm{Coef}_{AM}[A][M] + \mathrm{Coef}_{BM}[B][M] + \mathrm{Coef}_{ABM}[A][B][M])\} \quad (38)$$

or

$$v = \frac{V_{max}[A](1 + [M]/K_4)}{K_{mA}(1 + [M]/K_2) + [A][1 + (K_{mB}/[B])(1 + [M]/K_1) + [M]/K_3]} \quad (39)$$

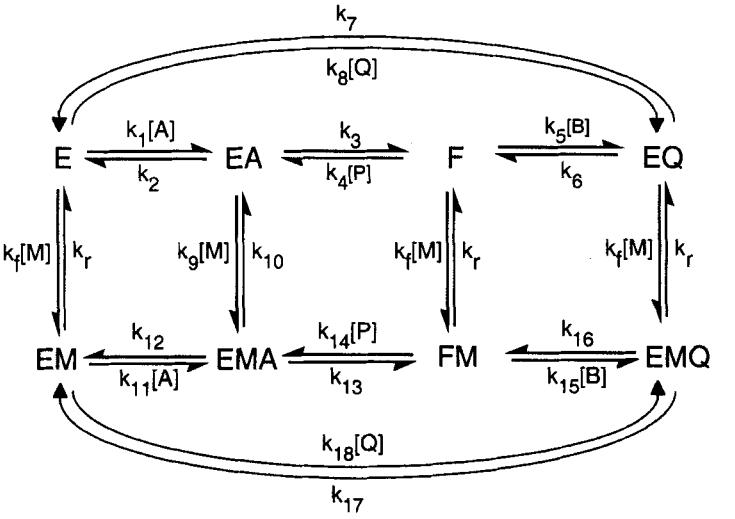

FIGURE 3 Reaction scheme for a ping pong bi bi reaction in the presence of an effector that binds to all enzyme species. The terms above and below the double arrows indicate the rate constant and ligand requirements for the reaction. EA and EMA represent, respectively, (EA + FP) and (EMA + FMP); EQ and EMQ represent, respectively, (FB + EQ) and (FMB + EMQ).

and

$$v = \frac{V_{max}[B](1 + [M]/K_4)}{K_{mB}(1 + [M]/K_1) + [B][1 + (K_{mA}/[A])(1 + [M]/K_2) + [M]/K_3]} \quad (40)$$

where K_{mA}, K_{mB}, and V_{max} are defined in the usual manner and

$$K_1 = \frac{Coef_A}{Coef_{AM}}, \quad K_2 = \frac{Coef_B}{Coef_{BM}},$$
$$K_3 = \frac{Coef_{AB}}{Coef_{ABM}}, \text{ and } K_4 = \frac{num_1}{num_2}$$

Note that the simplified equation contains only four constants associated with M. These inhibition (or activation) constants are related to the other limiting constants as shown below:

$$\frac{K_3}{K_2} = \frac{K'_{mA}}{K_{mA}}, \quad \frac{K_3}{K_1} = \frac{K'_{mB}}{K_{mB}}, \quad \frac{K_3}{K_4} = \frac{V'_{max}}{V_{max}}$$

Random AB Mechanism

The simplest way to analyze a random mechanism in the presence of a partial effector (Figure 4) is to assume that all ligand binding reactions are at equilibrium.[‡]

The velocity equation and the relationships between the various kinetic constants are identical to those shown earlier for the ordered mechanism, except that all K values are simple dissociation constants. The system can be analyzed as described by Segel[3] (see pp. 293–300).

SIMULATION

A velocity equation derived by the strict equilibrium method is an approximation. Consequently, the inhibition constants obtained by fitting experimental data to the approximate equation may not correspond to the dissociation constants for specific steps.[§] Nor do the number of experimental constants necessarily correspond to the number of M-binding reactions. Nevertheless, it was of interest to compare the experimental constants

[‡] In the absence of the partial effector, a less restrictive approach is to assume that only the ligands that add to free E are at equilibrium. Then the Cha method can be used to obtain a first degree velocity equation.[20]

[§] Even if the steady state velocity equation is exact, an experimental inhibition constant may not be a simple dissociation constant unless the effector is a dead end inhibitor.

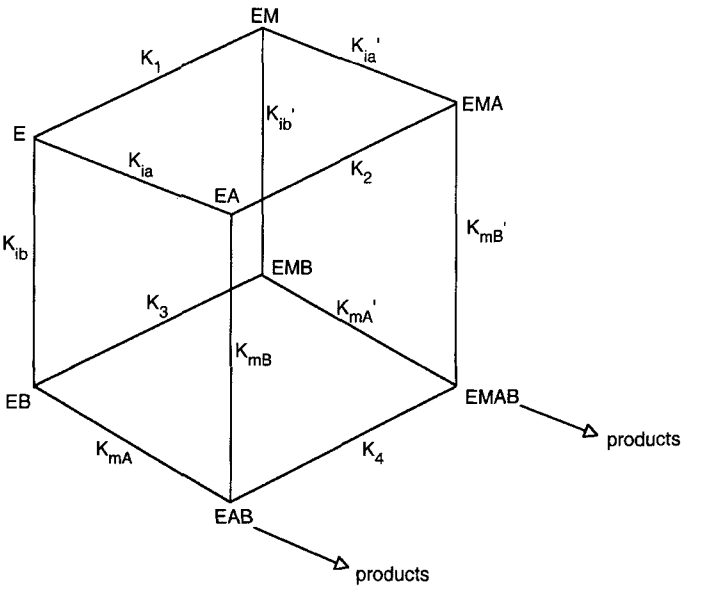

FIGURE 4 Reaction scheme for a rapid equilibrium random mechanism in the presence of an effector that binds to all enzyme species. All substrate kinetic constants and K_1 through K_4 are simple dissociation constants.

to actual values. A simulation based on the complete velocity equation for a steady state bireactant partial effector mechanism was beyond our computational means. But data for a unireactant partial inhibition mechanism (Figure 5) under steady state conditions could be generated. The steady state solutions for the four enzyme species (in the absence of products) are shown below. For clarity, only the numerators of the right-hand terms are shown. In each case, the denominator, $\sum$, equals the sum of the numerators of all the species

$$[\mathrm{E}]/[\mathrm{E}]_t = \{k_5(k_2+k_3)(k_7+k_9+k_{10}) + k_6k_9(k_2+k_3)[\mathrm{S}] + k_5k_8(k_7+k_{10})[\mathrm{I}]\}/\Sigma$$

$$[\mathrm{ES}]/[\mathrm{E}]_t = \{k_1k_5(k_7+k_9+k_{10})[\mathrm{S}] + k_1k_6k_9[\mathrm{S}]^2 + k_4k_6k_9[\mathrm{S}][\mathrm{I}]\}/\Sigma$$

$$[\mathrm{EI}]/[\mathrm{E}]_t = \{k_4(k_2+k_3)(k_7+k_9+k_{10})[\mathrm{I}] + k_1k_8(k_7+k_{10})[\mathrm{S}][\mathrm{I}] + k_4k_8(k_7+k_{10})[\mathrm{I}]^2\}/\Sigma$$

$$[\mathrm{EIS}]/[\mathrm{E}]_t = \{(k_2k_4k_6 + k_3k_4k_6 + k_1k_5k_8)[\mathrm{S}][\mathrm{I}] + k_1k_6k_8[\mathrm{S}]^2[\mathrm{I}] + k_4k_6k_8[\mathrm{S}][\mathrm{I}]^2\}/\Sigma$$

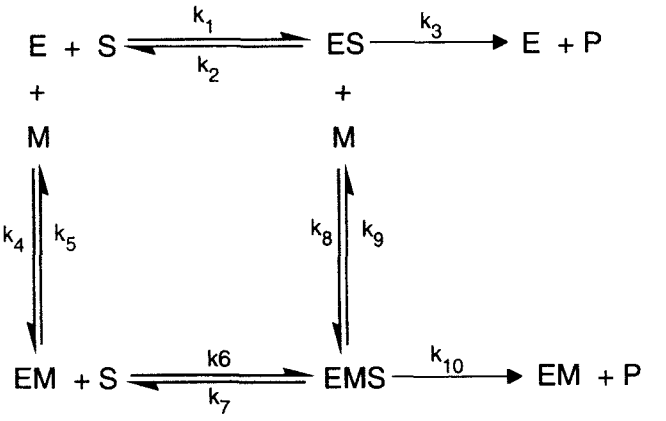

FIGURE 5 Reaction scheme for the unireactant general modifier mechanism. For the simulations shown in Figure 6, M is assumed to be a partial inhibitor.

Velocities were calculated from:

$$\frac{v}{[E]_t} = \frac{k_3[ES] + k_{10}[EIS]}{[E] + [ES] + [EI] + [EIS]} \tag{41}$$

Because the velocity equation for this mechanism contains terms in $[S]^2$, $[I]^2$, $[S]^2[I]$, and $[S][I]^2$ along with the usual constant, [S], [I], and [S][I] terms, some double reciprocal plots for subsaturating I may be curved over a broad 1/[S] range. The curvature may not be noticeable over the narrow range plotted although the best fitting straight lines may not intersect at a common point. The following criteria were used to assign the rate constants for simulations: (a) The values should be in the ranges for real enzymes.[16,17] (b) In order to avoid rapid equilibrium conditions, k_2 could not be $\gg k_3$. Also, k_7 could not be $\gg k_{10}$. Similarly, k_9 could not be $\gg k_{10}$ or k_7, nor could k_5 be $\gg k_6[S]$. (c) The ratio k_{10}/k_3 should be different from k_7/k_2 (to avoid reduction of the velocity equation to one that is first degree in [S] and [I]).[18,19] (d) The constants should be consistent with the Principle of Microscopic Reversibility: $k_1k_8k_7k_5 = k_4k_6k_9k_2$ and also (e) that $K_sK_i' = K_s'K_i$.

Figure 6(a) shows the double reciprocal plots for one of the simulations. The plots appear to be linear making this system a good candidate for analysis by curve fitting to a simplified velocity equation. Assuming that either of the I-binding reactions is at equilibrium, the method of Segel and Martin yields velocity Equation (42) which is identical to that obtained for rapid

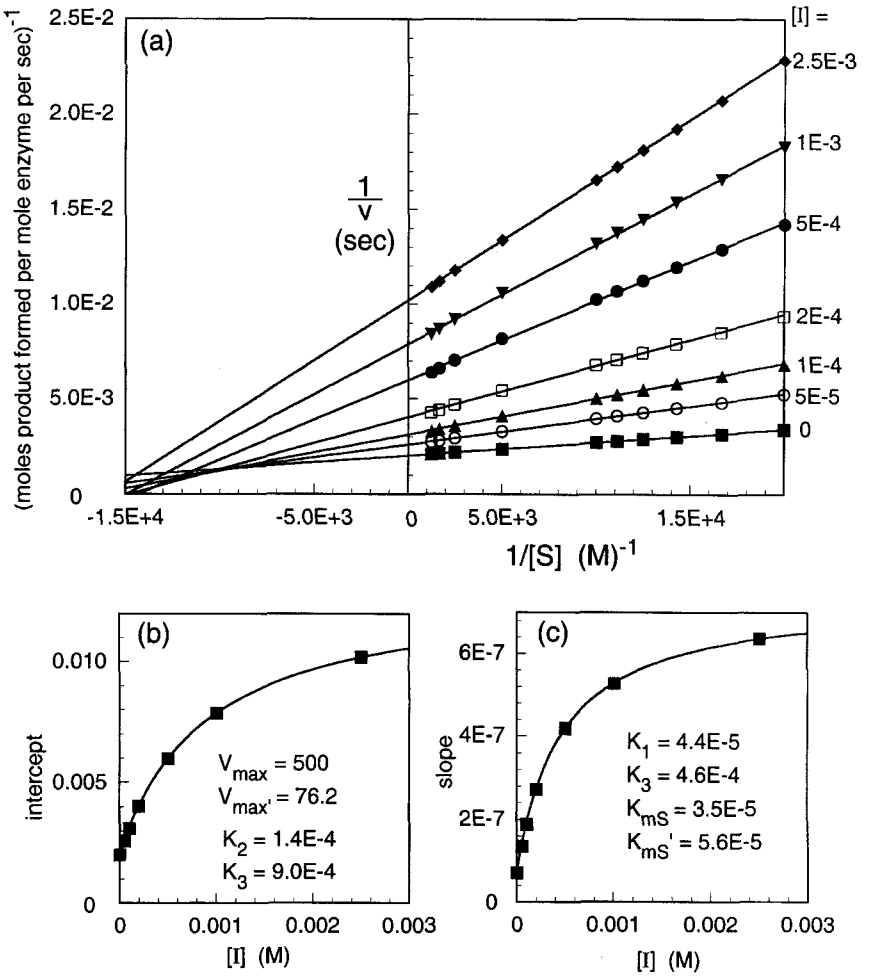

FIGURE 6 Double reciprocal plot and replots for a unireactant partial inhibition mechanism under steady state conditions. Data were generated from the complete velocity equation using the following rate constants: $k_1 = 2 \times 10^7$, $k_2 = 200$, $k_3 = 500$, $k_4 = 4 \times 10^6$, $k_5 = 200$, $k_6 = 3 \times 10^6$, $k_7 = 100$, $k_8 = 3 \times 10^5$, $k_9 = 50$, $k_{10} = 75$. Constants k_1, k_4, k_6, and k_8 are second order rate constants with units of $M^{-1} \times s^{-1}$. Constants k_2, k_3, k_5, k_7, k_9 and k_{10} are first order rate constants with units of s^{-1}. All concentrations are plotted in units of molarity. Velocity is plotted in terms of s^{-1} (i.e., moles of product formed per mole of enzyme per s). The kinetic constants calculated from the assigned rate constants are as follows: K_S (dissociation constant of ES) $= 10^{-5}$ M, K'_S (S dissociation constant of ESI) $= 3.33 \times 10^{-5}$ M, K_{mS} (Michaelis constant in the absence of I) $= 3.5 \times 10^{-5}$ M, K'_{mS} (Michaelis constant at saturating I) $= 5.83 \times 10^{-5}$ M, K_i (dissociation constant of EI) $= 5 \times 10^{-5}$ M, K'_i (I dissociation constant of ESI $= 1.67 \times 10^{-5}$ M, V_{max} (maximum velocity in the absence of I, i.e., k_{cat}) $= 500\,s^{-1}$, V'_{max} (maximum velocity at saturating I, i.e., k'_{cat}) $= 75\,s^{-1}$. The constants derived from the replots are shown within the figure.

equilibrium conditions. (This is not a general consequence of the method; it is true in the present case only because the system is unireactant.)

$$v = \frac{V_{max}[S](1 + [I]/K_3)}{K_{mS}(1 + [I]/K_1) + [S](1 + [I]/K_2)} \tag{42}$$

K_1, K_2 and K_3 are, in order, equivalent to K_i, αK_i, and $\alpha K_i/\beta$ in the alternate nomenclature often used for rapid equilibrium systems.[3] Constants were obtained by curve fitting the generated data to the following equations.[‖] (Equation (27) could also be used to obtain the limiting slope and intercept at saturating I.)

$$\text{intercept} = \frac{(1 + [I]/K_2)}{V_{max}(1 + [I]/K_3)} \quad \text{and}$$

$$\text{intercept} = \frac{(1 + K_2/[I])}{V'_{max}(1 + K_3/[I])} \tag{43}$$

$$\text{slope} = \frac{K_{mS}(1 + [I]/K_1)}{V_{max}(1 + [I]/K_3)} \quad \text{and}$$

$$\text{slope} = \frac{K'_{mS}(1 + K_1/[I])}{V'_{max}(1 + K_3/[I])} \tag{44}$$

The right-hand intercept and slope expressions are based on the relationships $V'_{max} = K_2 V_{max}/K_3$ and $K'_{mS} = K_2 K_{mS}/K_1$.

A separate plot of the control data over the [S] range 10^{-5}–10^{-4} M yielded the expected K_{mS} of 3.5×10^{-5} M and V_{max} of $500\,s^{-1}$ (i.e., 500 mol of product formed per mole enzyme per s.). The K_2 value of 1.4×10^{-4} M provided by the intercept replot (Figure 6(b)) is close to K'_i (1.7×10^{-4} M). A second intercept curve-fit returned a V'_{max} of $76.2\,s^{-1}$, which compares well with the true value of $75\,s^{-1}$. Curve fitting the slope replot (Figure 6(c)) with V_{max} entered as $500\,s^{-1}$ yielded $K_{mS} = 3.5 \times 10^{-5}$ M and $K_1 = 4.4 \times 10^{-5}$ M. The latter is in reasonable agreement with the value of K_i (5×10^{-5} M). A second slope replot with V'_{max} taken as $76.2\,s^{-1}$ yielded K'_{mS} as 5.6×10^{-5} M, in good agreement with the true value of 5.8×10^{-5} M. The returned K_3 values were 9×10^{-4} M (intercept replot) and 4.6×10^{-4} M (slope replot). Plotting a narrower [I] range (up to a maximum of 10^{-3} M)

‖ Curve fitting may be facilitated by substituting a very small finite value for $[I] = 0$. Entering the parenthetical terms in the form $(I + K_1)$ $(I + K_3)$ might also help.

and biasing the double reciprocal plots to intersect closer to a common point improved the agreement between the slope and intercept replots for K_3 with little effect on the values of K_1 and K_2. However, V'_{max} and K'_{mS} were increased by 10% and 20%, respectively. The cumulative results indicate that if the double reciprocal plots appear to be linear, fitting the data to the simplified velocity equation gives reasonable values for the kinetic constants.

DISCUSSION

The assumption of equilibrium between selected species is an approach that is often used to obtain velocity equations that would otherwise contain second (and higher) degree concentration terms. When the equilibrium approach of Cha[14] is applied to a bireactant partial inhibition or a nonessential activation mechanism, $[A]^2$ and $[B]^2$ terms are eliminated, but higher-ordered [M] terms remain. On the other hand, the method of Segel and Martin[15] provides first degree terms for all ligand concentrations. Why do the two different methods yield different velocity equations? After all, both approaches are based on the assumption of equilibrium for the same reactions. The answer is that the two methods use the equilibrium assumption in different ways. The Cha method assumes equilibrium within a reaction segment so that multiple substrate-binding species (e.g., E and EM) can be treated as a single species in the steady state. In contrast, the method of Segel and Martin is based on the concept that there is no net flux through reactions that are at equilibrium. That is, the latter approach assumes a strict equilibrium in which all EM formed from E + M *in the steady state* dissociates back to E + M.[¶] Both approaches are approximations which provide experimenters with a way to characterize and report the kinetics of a complex system in terms of familiar constants. One or the other approaches may have an advantage depending on the nature of the mechanism under consideration. For example, the Cha method yields a useful first degree equation for a random AB mechanism if it is assumed that the first substrate to bind in the forward direction is at equilibrium with its binary enzyme complex.[20] The method of Segel and Martin is useless for that system because it would eliminate obligatory substrate addition steps and result in a zero net reaction velocity. However, in the partial inhibition or nonessential activation mechanisms there is no requirement for a net flux through any

[¶] This subtle difference was not appreciated by Segel and Martin[15] and as a result, they mistakenly concluded that their method and the Cha method yielded the same final first degree equation. Varon *et al.*[24] subsequently pointed out the difference.

M-binding reaction after the presteady state period. In that case, the strict equilibrium method is not only applicable, but preferable because it yields a first degree equation containing obtainable constants that characterize the kinetic effects of M. Essentially, the final velocity equation is identical to the complete equation with all second degree and higher-ordered terms omitted. In this respect, the relationship between the complete velocity equation (or the Cha-derived equation) and the equation as derived by the method of Segel and Martin is similar to the relationship between the complete velocity or binding equation for a cooperative oligomer and the Hill equation (see Segel[3] pp. 360–361) – except in the latter case, it is the lower-ordered concentration terms that are omitted. As noted earlier, the final velocity equation derived by the strict equilibrium method is the same as that which would be obtained if M bound initially only to a single enzyme species. Because the same final equation is obtained regardless of which M-binding reaction is included in the steady state, separate experiments are needed to identify the actual M-containing species. Direct equilibrium binding[21,22] or inactivation protection methods[23] (under noncatalytic conditions) can be used for this purpose.

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