15

Three-Substrate Kinetics and Related Topics

INTRODUCTION

In Chap. 14 we extended the ideas of steady state and rapid equilibrium (first developed in Chap. 13) and applied them to various formal kinetic mechanisms for an enzyme with two substrates. The equations derived were put into the generalized Dalziel form of the initial rate equation. With the various two-substrate mechanisms we considered the use of experimentally determined initial rate parameters to distinguish among them, and came to the conclusion that to do this for certain mechanisms additional information is needed in the form of independently determined values of particular rate constants or equilibrium constants.

If instead of a two-substrate system we include a third substrate, it might intuitively be expected that this distinction is even more complex. As we will see, quite to the contrary, most three-substrate formal kinetic mechanisms can be identified on the basis of initial rate kinetic studies alone.

In this chapter we also consider two other situations where much information can be obtained from initial rate kinetic studies. Both are related to the general principles of three-substrate enzyme kinetics in that a third ligand is involved in an enzyme reaction with two substrates.

In the first situation, a regulatory ligand interacts with one or more of the enzyme complexes in a two-substrate ternary complex mechanism and alters the catalytic rate constant of the reaction. At nonsaturating concentrations of the regulatory ligand there are two forms of the enzyme ternary complex producing product, one with the regulatory ligand bound and the other with the normal ternary complex formed in the absence of the regulatory ligand. Initial rate studies can give a wealth of infor-

mation concerning the interaction of the regulatory ligand with various enzyme complexes.

In the second situation we examine various kinetic aspects of metal-ion-dependent enzymes. In these systems one of the major questions that must be answered is: What are the true substrates of the enzyme? It is quite possible that one or another of the substrates can only interact with the enzyme in the form of a metal complex.

Finally, in this chapter we examine briefly several other enzyme kinetic approaches that have been used. However, as will be discussed, these techniques, which involve systems where products are added as inhibitors or build up significantly during the course of the reaction, do not find the general usage that the other techniques we have discussed in Chaps. 13, 14, and in this chapter have.

THREE-SUBSTRATE KINETICS

As with two-substrate enzyme kinetics we can separate possible formal kinetic mechanisms into two classes. In the first, all the substrates must be bound prior to the appearance of product and the mechanism is known as "quaternary complex". In the second class, one or more products appear before all the substrates have bound and, as with the two-substrate systems, the mechanism is said to be "enzyme substituted." With each of these classes there are considerations regarding the order of substrate addition and whether or not various steps are in rapid equilibrium or must be treated using the less restrictive steady-state assumption.

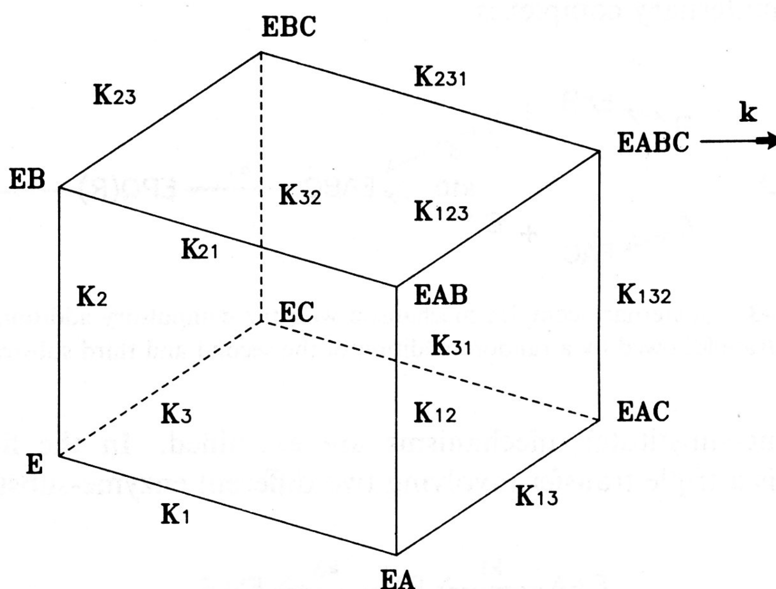

Figure 15-1 Random order of substrate addition in a three-substrate mechanism. The rapid-equilibrium assumption is made, allowing the various equilibrium constants to be included in the scheme. K_1, etc. are the equilibrium constants for A, etc. binding to free enzyme; K_{12}, etc. are the equilibrium constants for B, etc. binding to an EA-type complex; and K_{123}, etc. are the equilibrium constants for C, etc. binding to an EAB-type complex.

Formal Mechanisms

Five formal three-substrate mechanisms are considered. The first is the completely random-order addition of substrates in a quaternary complex mechanism, shown in Fig. 15-1. For the purposes of deriving a rate equation for this mechanism, the rapid-equilibrium assumption is made.

The second general mechanism considered is one involving a compulsory order of addition of each substrate, as shown in Fig. 15-2. Although this mechanism can be treated using either an equilibrium approach or the steady-state approach, for the purpose of this discussion we treat it using the steady-state assumption.

Figure 15-2 Compulsory order of subbstrate addition in a three-substrate mechanism.

The final quaternary complex mechanism to be considered is illustrated in Fig. 15-3. In this mechanism there is an obligatory first substrate followed by a random-order addition of the second and third. The addition of the first substrate is usually treated using the steady-state approach, while the random order of the subsequent substrate additions is handled by the rapid-equilibrium assumption. As with two-substrate random-order mechanisms such treatment assumes that the steps indicated as k_8 and k_{10} are much faster than the subsequent rate of catalytic interconversion of the quaternary complexes.

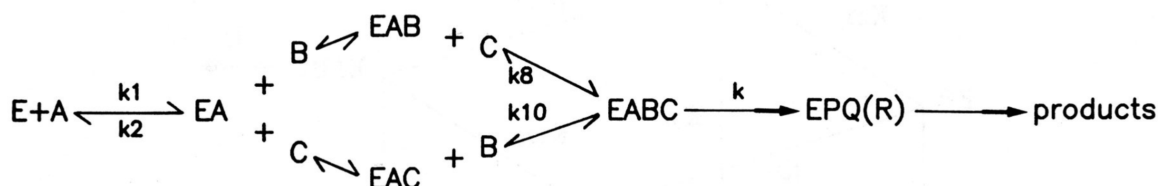

Figure 15-3 Quaternary complex mechanism with the compulsory addition of the first substrate followed by a random addition of the second and third substrates.

Two enzyme-substituted mechanisms are examined. In the first, shown in Fig. 15-4, there is a triple transfer involving two different enzyme-substituted species.

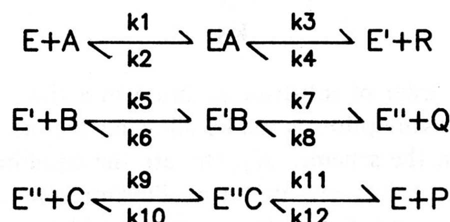

Figure 15-4 Enzyme-substituted mechanism for a three-substrate system involving a triple transfer.

In this mechanism there is an obligatory order of substrate addition and product release, and it is most simply treated by the steady-state approach.

In the other enzyme-substituted mechanism that we consider here, there is formation of a ternary EAB complex prior to formation of the enzyme-substituted intermediate (and release of the first product) and subsequent addition of the final substrate. The addition of the first two substrates can be ordered, as illustrated in Fig. 15-5, or random.

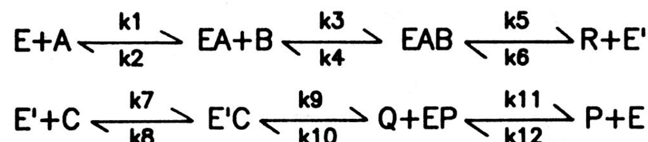

Figure 15-5 Enzyme-substituted mechanism with an ordered formation of a ternary complex prior to generation of the enzyme-substituted intermediate.

If the ternary complex formation is random ordered, the equilibrium approach can be employed, provided that the "off"-velocity constants are sufficiently rapid to allow the equilibrium condition to be reached. The ordered and random equilibrium versions of this mechanism give the same generalized rate equation.

Generalized Rate Equations

Each of the three substrate mechanisms mentioned can be represented by a generalized rate equation of the form given in

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{[A]} + \frac{\theta_2}{[B]} + \frac{\theta_3}{[C]} + \frac{\theta_{12}}{[A][B]} + \frac{\theta_{13}}{[A][C]} + \frac{\theta_{23}}{[B][C]} + \frac{\theta_{123}}{[A][B][C]} \quad (15\text{-}1)$$

Equation (15-1) can be rearranged to give

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_2}{[B]} + \frac{\theta_3}{[C]} + \frac{\theta_{33}}{[B][C]} + \left(\theta_1 + \frac{\theta_{12}}{[B]} + \frac{\theta_{13}}{[C]} + \frac{\theta_{123}}{[B][C]}\right)\frac{1}{[A]} \quad (15\text{-}1a)$$

which is in the form of a Lineweaver–Burk equation with A as the varied substrate at fixed concentration of the other two substrates, B and C. Equation (15-1) can, of course, be rearranged in a manner similar to that in Eq. (15-1a) to yield Lineweaver–Burk equations with either B or C as the varied substrate.

The only one of the five considered mechanisms to contain all eight terms of this equation is the rapid-equilibrium random-order mechanism of Fig. 15-1. The completely ordered mechanism (Fig. 15-2) lacks the θ_{13} term while the obligatory first substrate mechanism (Fig. 15-3) lacks the θ_{12} term and the θ_{13} term. Each of the quaternary complex mechanisms contains the θ_{123} term of Eq. (15-1).

In contrast, the various enzyme-substituted mechanisms (Figs. 15-4 and 15-5) each lack the θ_{123} term of Eq. (15-1). The triple transfer mechanism of Fig. 15-4 lacks four of the eight parameters; the θ_{12}, θ_{13}, and θ_{23} parameters are absent in addition to the θ_{123} parameter. The enzyme-substituted mechanism of Fig. 15-5 lacks the θ_{23} and θ_{13} parameters in addition to the θ_{123} parameter. In the discussion of Fig. 15-5 we considered the possibility that the ternary complex is formed by either

TABLE 15-1 Values of individual rate parameters for various three-substrate mechanisms

	Parameter							
Mechanism[a]	θ_0	θ_1	θ_2	θ_3	θ_{12}	θ_{13}	θ_{23}	θ_{123}
1	$\frac{1}{k}$	$\frac{K_{231}}{k}$	$\frac{K_{132}}{k}$	$\frac{K_{123}}{k}$	$\frac{K_{132} \cdot K_{31}}{k}$	$\frac{K_{123} \cdot K_{21}}{k}$	$\frac{K_{123} \cdot K_{12}}{k}$	$\frac{K_1 \cdot K_{12} \cdot K_{123}}{k}$
2		$\frac{1}{k_1}$	$\frac{1}{k_3}$	$\frac{Y}{k_5}$	$\frac{k_2}{k_1 \cdot k_3}$	0	$\frac{k_4 \cdot Y}{k_3 \cdot k_5}$	$\frac{k_2 \cdot k_4 \cdot Y}{k_1 \cdot k_3 \cdot k_5}$
3	$\frac{1}{k}$	$\frac{1}{k_1}$	$\frac{K_{132}}{k}$	$\frac{K_{123}}{k}$	0	0	$\frac{K_{123} \cdot K_{12}}{k}$	$\frac{K_2 \cdot K_{123} \cdot K_{12}}{k_1 \cdot k}$
4	$\frac{1}{k_3} + \frac{1}{k_7} + \frac{1}{k_{11}}$	$\frac{k_2 + k_3}{k_1 \cdot k_3}$	$\frac{k_6 + k_7}{k_5 \cdot k_7}$	$\frac{k_{10} + k_{11}}{k_9 \cdot k_{11}}$	0	0	0	0
5(a)	$\frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{11}}$	$\frac{1}{k_1}$	$\frac{k_4 + k_5}{k_3 \cdot k_5}$	$\frac{k_8 + k_9}{k_7 \cdot k_9}$	$\frac{k_2(k_4 + k_5)}{k_1 \cdot k_3 \cdot k_5}$	0	0	0
5(b)	$\frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{11}}$	$\frac{K_{21}}{k}$	$\frac{K_{12}}{k}$	$\frac{k_8 + k_9}{k_7 \cdot k_9}$	$\frac{K_1 \cdot K_{12}}{k_5}$	0	0	0

$$Y = \frac{k_6 \cdot k' + k_6 \cdot k_7 + k \cdot k_7}{kk_7}$$

[a] 1, Rapid equilibrium, random order; 2, compulsory order, steady state; 3, compulsory first substrate, random second and third; 4, triple transfer, enzyme substituted; 5, ternary complex, enzyme substituted: (a) compulsory, (b) random.

an ordered or a random addition of the first two substrates. The generalized form of the rate equation is identical in either case. The actual values of the individual rate parameters for each of these mechanisms are given in Table 15-1.

Determination of θ Parameters

The eight initial rate θ parameters of Eq. (15-1) are determined in a manner analogous to that described in Chap. 14 for the parameters in a two-substrate system. Primary Lineweaver–Burk plots are constructed with one of the three substrate concentrations varied at fixed concentrations of the other two.

The slope and intercept of such a Lineweaver–Burk plot with A as the varied substrate are functions of four parameters and the concentrations of the other two substrates.

Primary plot:

$$\text{slope} = \theta_1 + \frac{\theta_{12}}{[\mathrm{B}]} + \frac{\theta_{13}}{[\mathrm{C}]} + \frac{\theta_{123}}{[\mathrm{B}][\mathrm{C}]} \qquad (15\text{-}2a)$$

$$\text{intercept} = \theta_0 + \frac{\theta_2}{[\mathrm{B}]} + \frac{\theta_3}{[\mathrm{C}]} + \frac{\theta_{23}}{[\mathrm{B}][\mathrm{C}]} \qquad (15\text{-}2b)$$

The concentration of one of the two remaining substrates is varied and at each concentration a primary plot obtained. The slopes and intercepts are plotted in secondary plots as a function of the reciprocal of the second varied substrate concentration. Two secondary plots are obtained, one of the primary plot slopes and one of the primary plot intercepts. The slopes and intercepts of these secondary plots are given by

Secondary plot of slopes (primary) versus $1/[\mathrm{B}]$:

$$\text{slope} = \theta_{12} + \frac{\theta_{123}}{[\mathrm{C}]} \qquad (15\text{-}3a)$$

$$\text{intercept} = \theta_1 + \frac{\theta_{13}}{[\mathrm{C}]} \qquad (15\text{-}3b)$$

Secondary plot of intercepts (primary) versus $1/[\mathrm{B}]$:

$$\text{slope} = \theta_2 + \frac{\theta_{23}}{[\mathrm{C}]} \qquad (15\text{-}4a)$$

$$\text{intercept} = \theta_0 + \frac{\theta_3}{[\mathrm{C}]} \qquad (15\text{-}4b)$$

Each of the secondary plot slopes and intercepts is a function of two parameters from Eq. (15-1) and the concentration of the third substrate. Two sets of secondary plots are obtained with varied concentrations of the third substrate and tertiary plots

made of either slope or intercept from the appropriate secondary plot versus the reciprocal of the third substrate. Four tertiary plots are obtained and all eight initial rate parameters are determined from the appropriate slopes and intercepts.

Tertiary plots from secondary plots (intercepts):

(a) Secondary slopes:

$$\text{slope} = \theta_{23} \quad (15\text{-}5a)$$

$$\text{intercept} = \theta_2 \quad (15\text{-}5b)$$

(b) Secondary intercepts:

$$\text{slope} = \theta_3 \quad (15\text{-}5c)$$

$$\text{intercept} = \theta_0 \quad (15\text{-}5d)$$

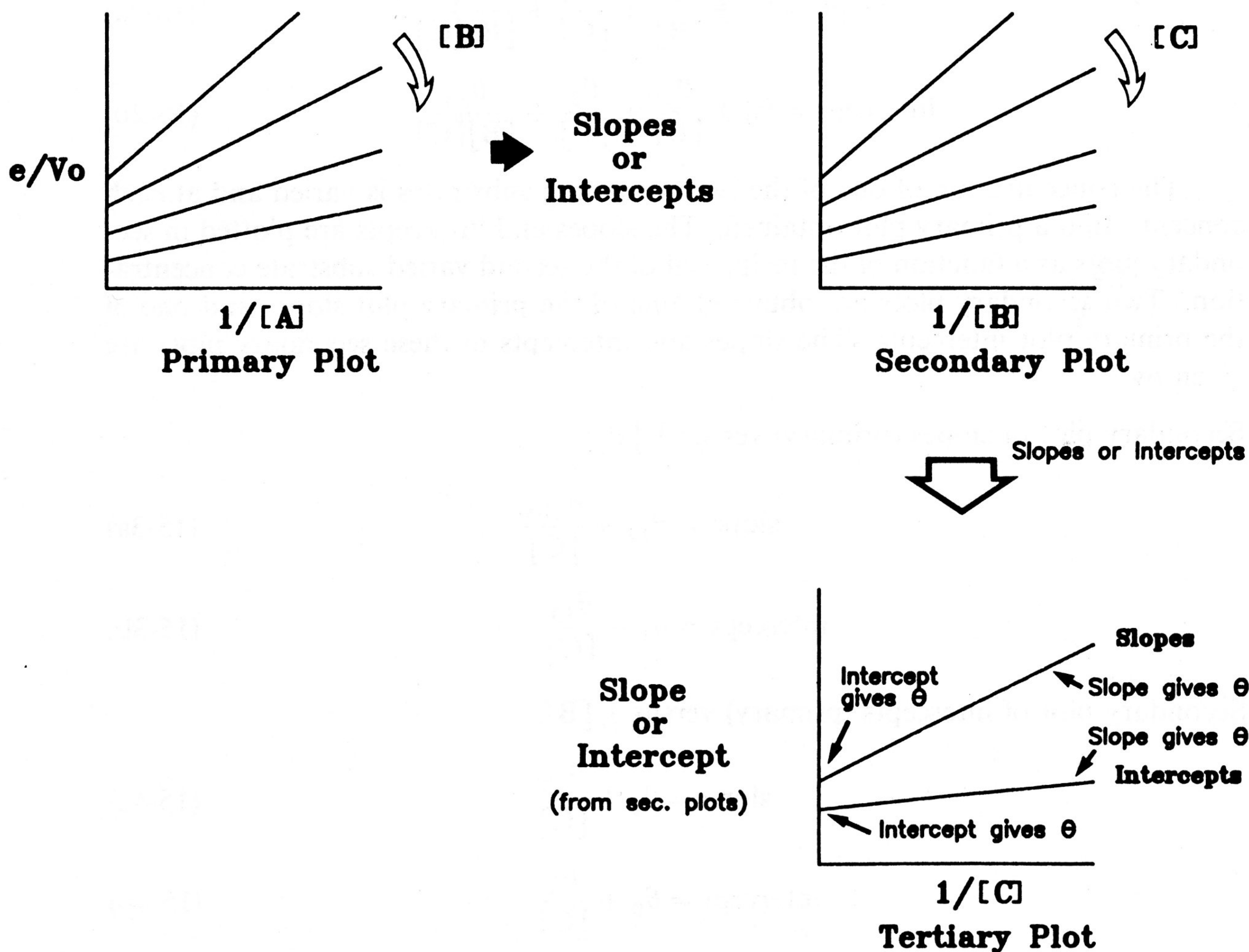

Figure 15-6 Outline of plots used to determine the initial rate parameters of Eq. (15-1).

Tertiary plots from secondary plots (slopes):

(a) Secondary slopes:

$$\text{slope} = \theta_{123} \tag{15-6a}$$

$$\text{intercept} = \theta_{12} \tag{15-6b}$$

(b) Secondary intercepts:

$$\text{slope} = \theta_{13} \tag{15-6c}$$

$$\text{intercept} = \theta_{1} \tag{15-6d}$$

The plotting procedures used to obtain the initial rate parameters are illustrated in Fig. 15-6.

Distinguishing Mechanisms

From Table 15-1 it is clear that each of the mechanisms we are examining in this chapter have different parameters from Eq. (15-1) equal to 0. The exceptions are mechanism I, where all the parameters have positive values, and mechanisms V_i and V_{ii}, both of which have the same parameters equal to zero.

By determining the parameters of Eq. (15-1), each of the mechanisms I to V can be identified. To distinguish mechanisms V_i from V_{ii}, approaches similar to those described in Chap. 14 are used. In practice it is often possible to distinguish several of the mechanisms from the patterns obtained in the primary or secondary plots without the necessity of obtaining all the data needed to construct the tertiary plots. This can lead to a considerable saving of material and effort since the number of data points needed to construct the tertiary plots is n^3 (where n is the number of points on each line) compared to n^2 data points for the secondary plots. Inspection of Eqs. (15-2) to (15-6) and Table 15-1 shows that, for example, mechanism IV gives quite distinctive primary plots.

Lineweaver–Burk plots with any substrate varied yield a series of parallel lines at various fixed concentrations of either of the other two substrates. Both variants of mechanism V give primary Lineweaver–Burk plots with A as the varied substrate having slopes that are affected by the concentration of B but not C, while the intercepts are affected by the concentration of either B or C. Thus a pattern of either intersecting or parallel primary plots is obtained depending on whether the concentration of B or C is altered at a fixed concentration of the other.

With mechanisms I, II, and III intersecting patterns of primary plots are always obtained due to the presence of θ_{123}. If one substrate is fixed *at a saturating concentration,* however, various terms in the rate equation are eliminated. For example, saturation with substrate C leads to the elimination of all terms containing the concentration of C—that is, θ_3, θ_{13}, θ_{23}, and θ_{123}. Mechanism III is now readily distinguished from mechanisms I and II by the existence of parallel lines in primary plots with A as the varied substrate at fixed concentrations of B. Similarly, if the

concentration of B is set at saturation, θ_2, θ_{12}, θ_{23}, and θ_{123} are eliminated and mechanisms I and II are easily distinguished.

The principal use of the determination of all the parameters in Eq. (15-1), apart from establishing the formal kinetic mechanism, is to find values for the various dissociation and rate constants involved in the mechanisms. As is apparent in Table 15-1, *all* the dissociation constants for mechanism I can be obtained from initial rate data and compared with independently determined values. Similarly, the rate constants of binding of the first and second substrates in the steady-state compulsory-order mechanism (mechanism II) can be obtained.

The Michaelis constants for the various substrates are given by the ratios θ_1/θ_0, θ_2/θ_0, and θ_3/θ_0 for substrates A, B, and C, respectively. In mechanism I these are the dissociation constants of the appropriate substrate from the quaternary complex EABC.

KINETICS OF TWO-SUBSTRATE SYSTEMS IN THE PRESENCE OF A REGULATOR

Many enzyme systems are subject to regulation by ligands other than the substrates. In such cases the regulatory ligand may activate or inhibit the reaction by binding at a site distant from the active site. An idea of the affinity the regulator has for the enzyme is often obtained by performing experiments at fixed substrate concentrations and varied regulator concentrations. Interpretation of this type of experiment assumes either that the regulator binds with equal affinity to all forms of the enzyme, or that it binds to only one form. Although these assumptions *may* be true in some systems, a far more complex situation is possible in a multisubstrate system.

The scheme illustrated in Fig. 15-7 represents a two-substrate random-order mechanism with a regulatory ligand, R, which can bind to both the binary complexes in the system. A rate equation for this scheme can be derived following the procedures outlined in Chaps. 13 and 14, with k_{cat}^1 as the rate constant for product production in the absence of regulator and k_{cat}^2 as the rate constant for product production in the presence of saturating amounts of the regulator. The regulator will be an activator if $k_{cat}^2 > k_{cat}^1$ and an inhibitor if $k_{cat}^2 < k_{cat}^1$. The rapid equilibrium rate equation derived making the rapid equilibrium assumption for this scheme is

$$\frac{e}{V_0} = \frac{K_{12} + K_{1R2} + K_{1R}[R]}{k_{cat}^1 \cdot K_{12} + k_{cat}^2 \cdot K_{1R2}[R]} + \frac{K_2(1 + K_{2R}[R])}{k_{cat}^1 \cdot K_{12} \cdot K_1 + k_{cat}^2 \cdot K_{1R2} \cdot K_{1R} \cdot K_1[R]}\frac{1}{A} + \frac{1 + K_{1R}[R]}{k_{cat}^1 \cdot K_{12} + k_{cat}^2 \cdot K_{1R2} \cdot K_{1R}[R]}\frac{1}{B} + \frac{1}{k_{cat}^1 \cdot K_{12} \cdot K_1 + k_{cat}^2 \cdot K_{1R2} \cdot K_{1R} \cdot K_1[R]}\frac{1}{[A][B]} \quad (15\text{-}7)$$

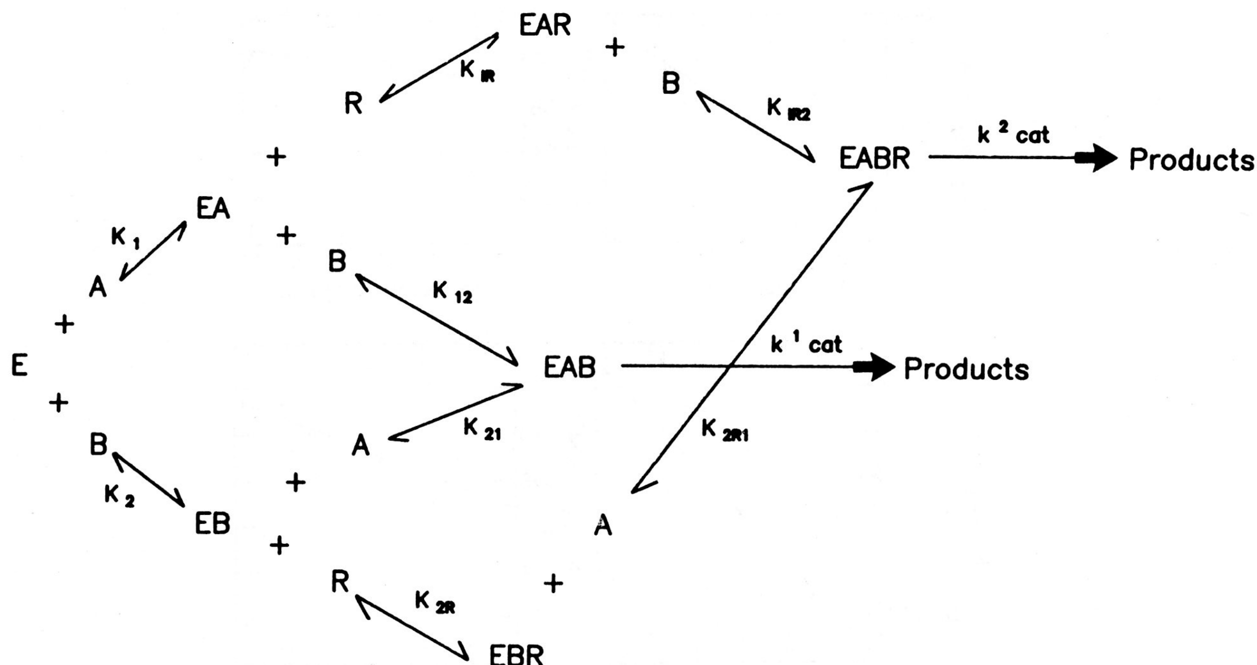

Figure 15-7 Rapid-equilibrium random-order scheme for an enzyme with two substrates and a regulatory ligand, R, which can bind to binary complexes.

From this equation, which fits the generalized rate equation for a two-substrate system, it is apparent that various ratios of parameters are functions of the concentration of the regulator, R, and dissociation constants in the mechanism. Specifically, θ_1/θ_{12} and θ_2/θ_{12} ratios are directly porportional to the concentration of R, as shown by

$$\frac{\theta_1}{\theta_{12}} = K_2(1 + K_{22}[\mathrm{R}]) \tag{15-8}$$

$$\frac{\theta_2}{\theta_{12}} = K_1(1 + K_{12}[\mathrm{R}]) \tag{15-9}$$

From these it is obvious that a plot of θ_1/θ_{12} or θ_2/θ_{12} versus [R] should be linear and allow calculation of the dissociation constants $K_{1\mathrm{R}}$ and $K_{2\mathrm{R}}$ from Fig. 15-7.

The galactosyl transferase reaction represents an excellent example of this approach. In the absence of the regulatory molecule α-lactalbumin, galactosyl transferase catalyzes the synthesis of *N*-acetyllactosamine from a sugar donor, UDD-galactose (U), and an acceptor substrate, *N*-acetylglucosamine (A). The reaction is inhibited by α-lactalbumin at high acceptor concentrations but activated at low acceptor concentrations. In the presence of α-lactalbumin the enzyme can also effectively utilize glucose as a substrate to give lactose. Figure 15-8 shows plots of ratios of θ parameters versus the concentration of α-lactalbumin which are clearly linear, as expected from Eqs. (15-8) and (15-9). Values of the dissociation constants for α-lactalbumin binding to the enzyme-acceptor or enzyme-donor complexes were also obtained.

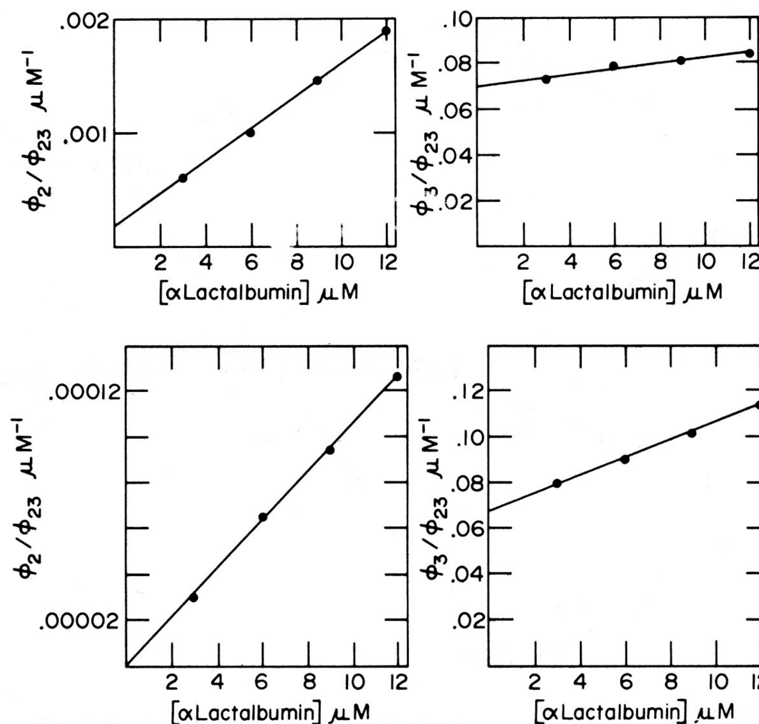

Figure 15-8 Plots of $\theta_2/\theta_{2,3}$ and $\theta_3/\theta_{2,3}$ versus [R] for galactosyl transferase using either *N*-acetylglucosamine (top) or glucose (bottom) as acceptor substrate. This enzyme has Mn^{2+} as a required independent first substrate. In the presence of saturating metal-ion concentrations, it can be regarded as a RERO two-substrate system.

With galactosyl transferase, the possibility that the regulatory ligand could also bind to free enzyme and ternary complex was considered, as shown in Fig. 15-9. Kinetic analysis of this scheme using Eq. (15-7) and data obtained from a covalently cross-linked transferase-α-lactalbumin complex allowed direct estimation of 10 of the 12 dissociation constants in this mechanism. The remaining constants, K_{1R} and

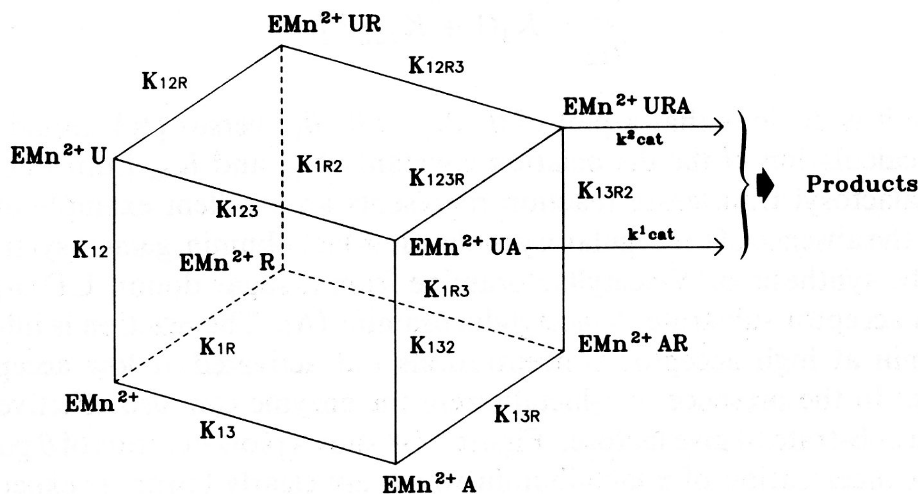

Figure 15-9 Scheme for galactosyl transferase-regulator interactions where the regulator can bind to free enzyme and the $E \cdot Mn^{2+} \cdot UA$ complexes.

Table 15-2 Dissociation constants for the mechanism shown in Figures 15-8 and 15-9

Constant	From initial rate data (μM)	From inhibition experiments (μM)	Independently determined (μM)
$K_{12}(\theta_{23}/\theta_3)$	13.7, 15.0, 10.1		14, 16
$K_{13}(\theta_{23}/\theta_2)$	6,600	4,800	5,000
$K_{123}(\theta_3/\theta_0)$	11,000	16,000	
$K_{132}(\theta_2/\theta_0)$	23		
K_{13R}	1.1	2.0	3.5
K_{12R}	50, 17.6	30, 35	17.7

K_{123R}, were calculated assuming overall equilibrium and were 555 μM and 0.3 μM, respectively. Table 15-2 shows values for some of the constants obtained from kinetic measurements together with comparable constants determined by independent methods. A comparison of the values of K_{1R}, K_{12R}, K_{13R}, and K_{123R} proves that the principal driving force for the regulator binding is the presence of the acceptor in the enzyme complex even though the regulator can bind to all the enzyme species given in Fig. 15-9.

Although this is a single example, it does illustrate the amount of information that can be obtained from initial rate kinetic studies of a system involving two substrates and a regulatory ligand. The equations here apply to the random-order equilibrium situation; this is the most complex situation that might be encountered. In compulsory-order systems, although the initial rate equations are more complex, the interpretation of the kinetic effects of a regulatory ligand are straightforward.

KINETICS OF METAL-DEPENDENT SYSTEMS

A number of enzymes have a required metal ion. In some instances it may be involved only in maintaining the structural integrity of the protein and is not involved directly in ligand binding or catalysis. In other cases the metal ion may be an absolutely required participant in the reaction; if so, and if the metal ion is reversibly bound to the protein, it must be considered as a substrate. The final possibility to consider is that the metal ion may not be required for reaction but acts as a regulatory ligand, and is considered as described in the preceding section.

When the metal ion is a reversibly bound, required participant in the reaction, several possibilities can be envisaged. The metal ion may bind only to the enzyme, in which case it can be regarded as a true third substrate and treated in exactly the manner described earlier for a three-substrate system. It is quite likely, however, that the metal can bind to one or other of the remaining substrates and the enzyme. In such a case the question that must be asked is: What is the true substrate of the enzyme, the free substrate or the metal–substrate complex? It is possible that the metal–substrate complex is the only form of the substrate to bind effectively to the enzyme,

while the free substrate acts as an inhibitor (or vice versa). If the dissociation constants of the metal substrate complexes are known or can be determined, it is possible to resolve this question. Resolution depends on two assumptions: (1) the reaction velocity is represented by a first-degree equation in the concentrations of the true substrates, and (2) the metal–substrate complex is kinetically distinct from the free substrate. In a two-substrate plus metal-ion system where the metal ion interacts with only one of the substrates, it is a simple process to calculate the concentrations of free and metal-complexed substrate and plot the appropriate Lineweaver–Burk plots. If the metal ion complexes with both substrates, then changing any one of the participants affects the concentration of the remaining reactants and must be taken into account.

When initial rate data are plotted as a Lineweaver–Burk plot versus the total metal-ion concentration, an apparent substrate inhibition is seen if the true substrates are the free ligands, because increased metal-ion concentrations lead to an effective decrease of the true substrate concentration.

EFFECTS OF PRODUCTS

As indicated in the introduction to this chapter, there are various other initial rate kinetic techniques that we have not covered in detail in Chaps. 13 to 15. As emphasized, initial rate kinetics studies are best used in the context of eliminating possible kinetic mechanisms rather than in proving a particular mechanism. This is true because it is always possible to make a mechanism more complex to accommodate the experimentally obtained data. The two occasionally used initial rate studies described briefly in this section are inherently more complex than the other kinetic approaches described in this book, and as a result are not as easily interpreted.

As we have derived equations in Chaps. 13 and 14 to describe the dependence of the *initial* rate on substrate concentrations, it is assumed that product molecules are initially absent, and as a result, steps involving product *binding* (rather than release) can be ignored. Two types of experimental situations where this is *not* the case are sometimes employed. In the first, product molecules are added at fixed concentrations prior to reaction initiation by addition of enzyme. Since the product can bind to the enzyme to give E-P complexes, which are not on the pathway of substrates being converted to product, an inhibition of S-to-P conversion results. This is known as *product inhibition*, and its type depends on a variety of factors: Does the added product simply withdraw enzyme from otherwise productive catalysis, can the product act as a substrate analog (in which case it can be considered in the same way as described in Chap. 15 for substrate analogs), or can it form abortive E-S-P complexes? Rate equations for these various situations are easily obtained using the approaches described earlier, but of course are far more complex. This makes product inhibition studies of limited use in establishing a formal kinetic mechanism for an enzyme. Once a formal mechanism is known, however, these studies can be vital in establishing what types of "abortive" complexes exist in a mechanism. Such complexes are discussed further in Chap. 16.

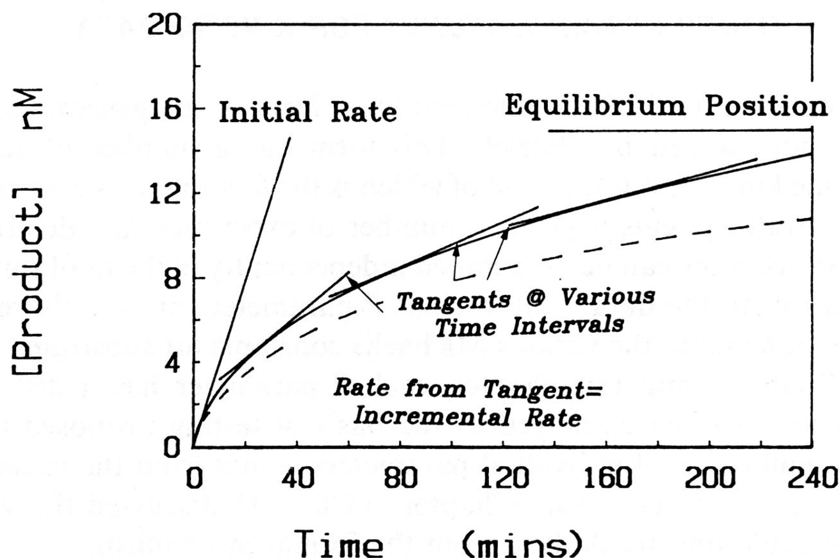

Figure 15-10 Full-time-course analysis of product accumulation. Product concentration is monitored as a function of time after addition of enzyme to a reaction mixture. The monitoring is continued until the equilibrium concentration of product is approached. Indicated on the graph is the initial rate and the incremental rate at some time point where product buildup effects are negligible. The incremental rate is determined solely by the decrease in substrate concentration. Also shown (---) is a projected full time course where product inhibition is seen.

The second experimental situation involves a *full time-course* analysis of an enzyme-catalyzed reaction rather than an initial rate study. In its simplest conception this analysis (where the amount of product is monitored over a much longer period than that in an *initial* rate study) offers the chance of obtaining a variety of parameters from a single experiment. Consider the situation in the reaction mixture once the reaction is initiated. At time $t = 0$, a true initial rate at a particular substrate concentration (the starting concentration) is obtained. At later time points the rate—as judged by the slope of the tangent to the time course (see Fig. 15-10)—is governed by a variety of considerations.

First, the substrate concentration at time point t is less than existed at $t = 0$, due to the reaction proceeding (as t increases, the substrate concentration continues to decrease until equilibrium is reached). Second, product molecules are now present and cause product inhibition and possibly abortive complex formation (as with decreasing substrate concentrations, the product concentration increases with time).

If product inhibition effects are insignificant a full-time-course analysis allows K_m and $V_{\max}$ to be obtained directly since the incremental rate shown in Fig. 15-10 is directly proportional to the incremental substrate concentration, as related by the Michaelis–Menton equation. In reality, of course, such a simple situation does not hold in the majority of cases and the analysis is far from simple since product inhibition with a continuously changing concentration of product must also be taken into account. At this point the complexity of the rate equation and the experimental accuracy of the data often preclude meaningful analysis.

OTHER NOMENCLATURES FOR KINETIC DATA

In this book we have used the double reciprocal form of the generalized initial rate equation first popularized by Dalziel. This form has a number of advantages in practical enzyme kinetics, not the least of which is the fact that it is easy to remember. With multisubstrate systems it yields a number of experimentally determined θ parameters whose accuracy can be determined independently of the fit of some proposed kinetic mechanism to the data. The various θ parameters are simply related to the V_{max} of the reaction and to the various Michaelis constants for substrates. As we have discussed in Chap. 14 and this chapter, each θ parameter has a distinct physical significance which is often employed as the basis of testing proposed mechanisms. The physical significance of individual parameters comes from the initial rate equation for the proposed mechanism. Chapters 14 and 15 discussed the various ways in which these equations are derived from the formal mechanism.

Over the years various nomenclatures for the constants that can be obtained from initial rate studies have been used. Apart from the Dalziel system two others are worthy of mention since they too are frequently employed in the literature—those of Alberty and Cleland. Table 15-3 lists these constants for a two-substrate system in terms of the Michaelis constant for each substrate, the dissociation constant for the first substrate in a ternary complex mechanism and the maximum velocity of the reaction. Although each of these constants has the same physical significance for a particular mechanism, the Dalziel parameters have the additional advantage that each K_m is the ratio of two initial rate parameters with individual physical significances as described in Chap. 14 and this chapter, whereas in the Alberty and Cleland nomenclatures the K_m values are individual parameters.

The final comments that must be made at this point are in regard to experimental design of enzyme initial rate kinetic studies. With one exception, all the formal kinetic mechanisms considered in Chaps. 13 to 15 provide initial rate equations predicting linear Lineweaver–Burk plots. In designing a kinetic study of an enzyme it is, however, important that as wide a range of substrate concentrations be used as is feasible.

TABLE 15-3 Kinetic constants for two-substrate reactions using the nomenclature of Dalziel, Alberty, and Cleland

Constant	Dalziel	Alberty	Cleland
K_m(first substrate)	$\frac{\theta_1}{\theta_0}$	K_A	K_a
K_m(second substrate)	$\frac{\theta_2}{\theta_0}$	K_B	K_b
K_d(first substrate)	$\frac{\theta_{12}}{\theta_2}$	$\frac{K_{AB}}{K_B}$	K_{ia}
V_{max}	$\frac{e}{\theta_0}$	V_f	V_1

This not only makes it possible to experimentally demonstrate linearity of Lineweaver–Burk plots, but it means that all the terms in the Dalziel generalized rate equation can be determined. To ensure this, it is necessary to make measurements at low substrate concentrations, where all the terms in the rate equation play significant roles in governing the rate.

At the other end of the concentration scale, many of the types of nonlinearity in Lineweaver–Burk plots that we discuss in Chap. 17 are clearly observable only at relatively high substrate concentrations—hence the need to use a wide range of substrate concentrations.