19

Use of Isotopes in Enzyme Kinetic Mechanism Analysis

INTRODUCTION

The use of isotopically labeled compounds has led to a number of advances in enzyme kinetic analysis. These can be grouped into four categories, one of which was discussed in Chap. 1: the use of isotopically labeled substrates to follow the reaction rate. In such cases the isotope in the substrate is transferred to product and its amount quantitated at various time intervals to estimate the *rate*. The other three categories of information obtainable from experiments using isotopically labeled substrates involve (1) the formal kinetic mechanism of a particular enzyme-catalyzed reaction, (2) non-rate-limiting steps in the formal kinetic mechanism, and (3) identification of bond-breaking steps with particular phases of the formal kinetic mechanism of a reaction.

In this chapter we examine a variety of techniques employing isotopically labeled substrates which provide information that can complement that obtained from initial rate or rapid-reaction studies and, in many cases, examine ambiguities arising from initial rate kinetic studies.

ISOTOPIC COMPOUNDS AS ALTERNATIVE SUBSTRATES

In earlier chapters we considered the use of alternative substrates in initial rate studies as a method for elucidating the formal kinetic mechanism of a reaction. One problem frequently encountered is when no *good* alternative substrate is available. Although many enzymes can use alternative substrates, it is often found that they

are very much poorer than the true substrate, and as a result one is left with a question as to whether the alternative follows the same formal kinetic mechanism. One way out of this dilemma is to use a radioactive label in the substrate and to mix labeled and nonlabeled substrates and follow the rate of reaction with one or the other. It is important to be able to follow the rate with only one of the alternate substrates. So that the isotopically labeled substrate does not alter the formal kinetic mechanism, care should be taken that the isotope does not involve any bond that may be broken during the course of the reaction; otherwise, an isotope effect may alter rate-limiting steps in the overall mechanism.

If we examine two situations, a compulsory-order two-substrate mechanism and a random-order, equilibrium two-substrate mechanism, Eqs. (19-1) to (19-3) describe the use of isotopically labeled alternative substrates.

Compulsory-Order Mechanism

The mechanism, in the presence of the normal substrate, S, and the isotopically labeled substrate, S_1^*, is given in Fig. 19-1. The rate equation when S_1, S_1^*, and S_2 are present is given by

$$\frac{e}{V_0} = \phi_0 + \frac{\phi_1}{S_1}(1 + X + Y + Z) + \frac{\phi_2}{S_2} + \frac{\phi_{12}}{S_1S_2}(1 + X + Y + Z) \qquad (19\text{-}1)$$

where

$$X = \frac{k'_1S_1^*(k'_4 + k'_5)}{k'_2(k'_4 + k'_5) + k'_3k'_5(S_2)}$$

$$Y = \frac{k'_3[S_2]X}{k'_4 + k'_5}$$

$$Z = \frac{k'_3k'_5[S_2]X}{k'_7(k'_4 + k'_5)}$$

and where k' and so on indicate steps involving the isotope. Thus S_1^* is a competitive inhibitor versus S_1, *but* relative to S_2, the inhibition is parabolic.

If an isotope of S_2 is available (S_2^*) such that the steps

$$ES_1 + S_2^* \longrightarrow EP_1P_2^* \longrightarrow EP_1 + P_2^*$$

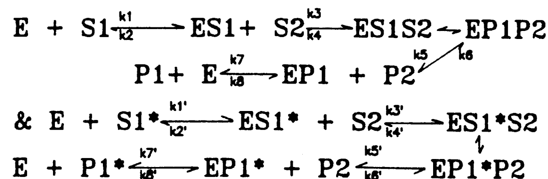


Figure 19-1 Compulsory-order mechanism in the presence of an isotopically labeled S_1^* alternative substrate.

must be included, the rate equation has to be modified:

$$\frac{e}{V_0} = \phi_0 + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2}\left[1 + \frac{k'_3 S_2^*}{k'_4 + k'_5}\left(1 + \frac{k'_5}{k'_7}\right)\right] + \frac{\phi_{12}}{S_1 S_2}\left(1 + \frac{k'_3 k'_5 S_2^*}{k_2(k'_4 + k'_5)}\right) \quad (19\text{-}2)$$

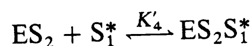
and now the inhibitor is competitive versus S_2 but noncompetitive versus S_1.

Random-Order, Rapid-Equilibrium Mechanism

As with the equations for a simple random-order, rapid-equilibrium (RORE) mechanism, those in the presence of isotopically labeled alternative substrate are easy to derive and are symmetrically arranged.

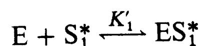
$$\frac{e}{V_0} = \phi_0 + \frac{\phi_1}{S_1}\left(1 + \frac{S_1^*}{K'_4}\right) + \frac{\phi_2}{S_2} + \frac{\phi_{12}}{S_1 S_2}\left(1 + \frac{S_1^*}{K'_1}\right) \quad (19\text{-}3)$$

where K'_4 is



$$ES_2 + S_1^* \xrightarrow{K'_4} ES_2S_1^*$$

and K'_1 is



$$E + S_1^* \xrightarrow{K'_1} ES_1^*$$

Consequently, inhibition by S_1^* is *competitive* versus S_1 and noncompetitive versus S_2. With an isotopically labeled analog of S_2, S_2^* is competitive versus S_2 and noncompetitive versus S_1. Two examples illustrate the uses of such isotopically labeled alternative substrates.

Alcohol Dehydrogenase. In this system NAD and [^{14}C]-NAD are used as alternative substrates and Lineweaver–Burk plots drawn with either NAD or ethanol as the varied substrate. As shown in Fig. 19-2A, when [^{14}C]-NAD is examined as an inhibitor with respect to NAD, competitive inhibition results. When, however, [^{14}C]NAD is used as an inhibitor with respect to ethanol (Fig. 19-2B), the inhibition is parabolic. These results are consistent with a compulsory-order mechanism having NAD as the first substrate. Incidentally, this experiment can also be done using the thionicotinamide analog of NAD, which has an S-NAD $\rightleftharpoons$ S-NADH *isosbestic* point at 342 nm—thus measuring at 342 nm measures *only* NADH production as required.

Hexokinase. This enzyme catalyzes the phosphorylation of glucose by ATP in a magnesium-dependent reaction. [^{14}C]- and [^{12}C]glucose are convenient alternative substrates, and the following experiments explore the kinetic mechanism of the reaction.

As shown in Fig. 19-3 (left), [^{12}C]glucose is a competitive inhibitor with respect to [^{14}C]glucose when the assay is conducted by following ^{14}C transfer into product. When [^{12}C]glucose is used as an inhibitor versus the other substrate, MgATP, a linear, noncompetitive inhibition is observed. The experimental data are *not* consis-

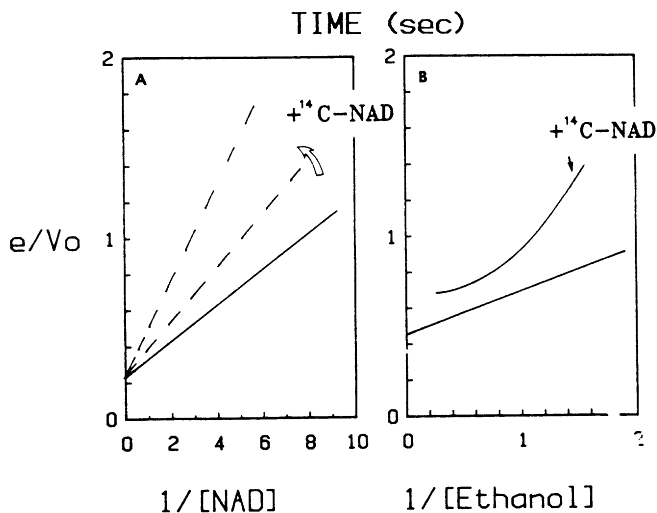


Figure 19-2 Experiments with alcohol dehydrogenase: initial rate of NADH production followed in the absence or presence (----) of [^{14}C]NAD. Varied substrate is [^{12}C]NAD in part (A) or ethanol in part (B). Only reduction of [^{12}C]NAD is followed.

tent with a simple compulsory order of substrate addition with glucose as the first substrate, but are consistent with either a rapid-equilibrium, random-order mechanism or a compulsory-order mechanism with MgATP as the first substrate. Experiments with isotopically labeled ATP as an alternative substrate versus ATP would, of course, distinguish between these possibilities.

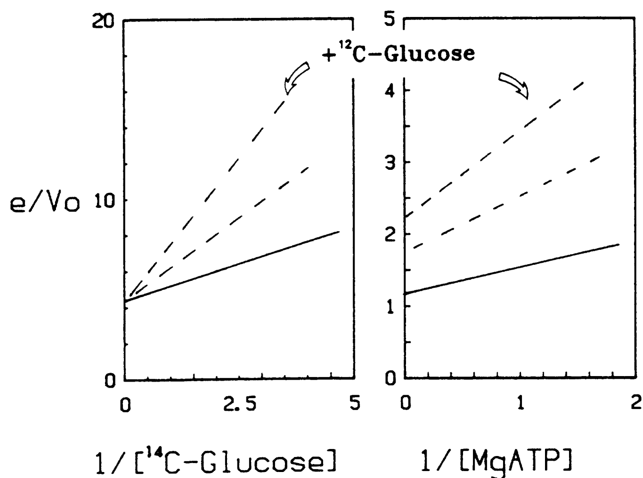


Figure 19-3 Experiments with hexokinase: initial rate of [^{14}C]glucose transfer into product is followed in the absence or presence (----) of [^{12}C]glucose. Varied substrate is [^{14}C]glucose in part (A) of MgATP in part (B).

Glucose–Fructose + Enzyme
Glucose–Enzyme + Fructose
+Pi
Enzyme + Glucose–1–Phosphate

Figure 19-4 Enzyme-substituted mechanism for sucrose phosphorylation.

ISOTOPE EXCHANGE AT EQUILIBRIUM

Isotope-exchange studies have long been used to examine formal kinetic mechanisms of enzyme-catalyzed reactions. Early studies of isotope exchange examined if an enzyme exhibited an enzyme-substituted (ping-pong) mechanism. In such a mechanism, represented as

$$E + XG \rightleftharpoons EXG \rightleftharpoons EG + X$$

$$EG + Y \rightleftharpoons EYG \rightleftharpoons E + YG$$

the use of radioactively labeled X and nonlabeled XG allows an exchange of isotope into XG *in the absence* of Y. Such an isotope exchange is not possible in a ternary complex mechanism. Several examples illustrate the sort of observations that have been made.

In the reaction catalyzed by sucrose phosphorylase,

$$\text{glucose-fructose} + P_i \rightleftharpoons \text{G-1-P} + \text{fructose}$$

an isotope exchange between $^{32}P_i$ and glucose-1-phosphate is observed in the absence of fructose, suggesting that the enzyme operates via the enzyme-substituted mechanism illustrated in Fig. 19-4.

The biotin-dependent carboxylases, which catalyze the overall reaction,

$$\text{ATP} + HCO_3^- + \text{proprionyl-CoA} \xrightleftharpoons{Mg^{2+}} \text{ADP} + P_i + \text{meth. malonyl-CoA}$$

catalyze an exchange between ATP and ^{32}Pi, but it occurs only in the presence of HCO_3^- and Mg^{2+}. This suggests that an enzyme-substituted mechanism is in fact operating and that it involves the stages shown in Fig. 19-5.

One of the problems with this type of experiment is that the presence of a small contaminant of the second substrate allows the "exchange" to occur in the absence of *added* second substrate. In some cases small amounts of *tightly bound* second substrate on the enzyme are sufficient to allow the "exchange" to take place, when in

ATP + HCO_3 + ENZ ⇨ CO_2ENZ + ADP
+Pi
+PropCoA
Meth.MalonylCoA + ENZ

Figure 19-5 Enzyme-substituted mechanism for a biotin-dependent carboxylase.

reality it results from the simple bulk transfer of substrate to product via a ternary complex mechanism rather than from the existence of an isotope exchange resulting from an enzyme-substituted mechanism.

Isotope-exchange-at-equilibrium studies are usually directed at a variety of questions asked about ternary complex mechanisms. This approach is a powerful (but as we will see, somewhat tedious) tool for distinguishing among ternary complex mechanisms, for examining the existence of abortive complexes in mechanisms, for obtaining information on non-rate-limiting steps in reaction mechanisms, and for ascertaining the true equilibrium nature of a rapid-equilibrium, random-order mechanism.

The principle of the approach is quite simple. An enzyme is mixed with substrates (or products, or a mixture of substrates and products) at a particular concentration and the reaction is allowed to proceed until chemical equilibrium is reached. At this point a small amount of isotopically labeled substrate (or product) is added and the *rate of isotope exchange* into the appropriate product measured. Because the small amount of isotopically labeled substrate (or product) is chosen such that it does not significantly displace the equilibrium position of the reactants, the rate of isotope exchange is measured *at equilibrium*. The exchange rate under such conditions can be regarded as a function of the concentration of the enzyme complex containing the substrate whose exchange rate is being monitored. As with other rate measurements, the dependence of the rate on the saturation of the enzyme with reactants is followed, bearing in mind that individual reactant concentrations cannot be altered in isolation; otherwise, a perturbation of the equilibrium results. When the concentration of a particular substrate is changed, the concentration of a product must also be altered so that the equilibrium position is not changed.

In a typical two-substrate system, various exchanges are possible, depending on the chemical nature of the reactants; thus a number of different exchanges can be followed. For the reaction $A + B \rightleftharpoons P + Q$, the following exchanges are possible: $A \rightarrow P$, $A \rightarrow Q$, $B \rightarrow P$, and $B \rightarrow Q$.

Let us consider first the case of a compulsory-order reaction, represented schematically in Fig. 19-6. For such a mechanism we can consider the effects of raising various reactant concentrations on the possible exchange rates.

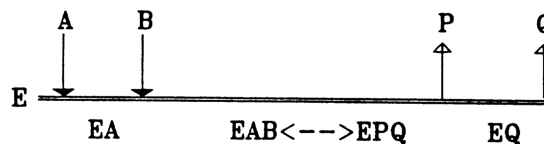


Figure 19-6 Schematic representation for a compulsory-order mechanism.

Exchanges Involving A

If an exchange of isotope *from* A is considered, the only way that the isotope of A (A^*) can enter the system to exchange into P or Q involves exchange of A^* with A in the complex EA. Any action that *decreases* the concentration of the complex EA

decreases the rate of exchanges involving A* (i.e., A* → P or A* → Q). If the concentration of A is raised at a fixed concentration of B, the concentration of EA will increase to a maximum and exchanges involving A* will increase to a maximum. If, however, the concentration of B is raised at a fixed concentration of A, the concentration of EA will decrease eventually to zero, as all of the EA complex is bound by B and pushed into the central EAB ⇌ EPQ complexes. At this point *no* exchange involving A* is possible and the *rate* of exchanges involving A* falls to zero. Since the reaction scheme is symmetrical, similar effects of raising the concentration of P on exchanges involving Q* is observed.

In this example, not only the *dependence* of exchange rate on reactant concentration but also the *maximum rate* of exchange may differ, depending on the exchanges followed. In compulsory-order mechanisms the overall rate-limiting step is often a product release step. Consider what one expects to observe if the release of Q is rate limiting. Exchanges that do not involve Q occur at a faster maximum rate than those involving Q. Alternatively, if the overall rate-limiting step is release of P, all of the exchanges have the same maximum rate since all exchange routes depend on the release of P.

In a rapid-equilibrium, random-order mechanism, the pattern of dependence of the exchange rate on reactant concentration is easily predictable. Because all complexes in the substrate addition side of the reaction (or for that matter on the product release side) are in equilibrium, and because either substrate (A* or B*) has two routes of entry into an exchange (by combination with E or by combination with the appropriate binary complex), all possible exchange rates rise to a maximum as any

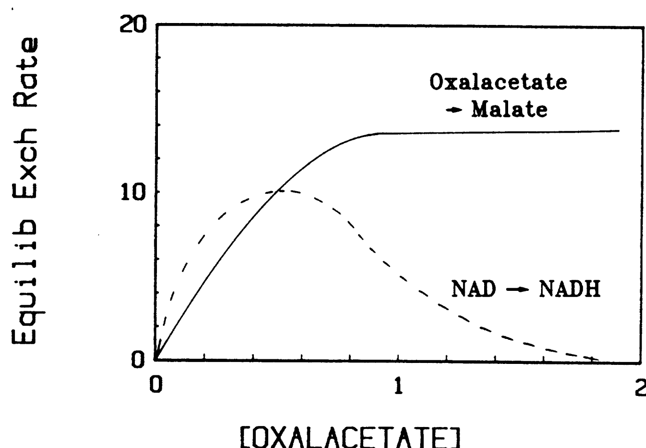
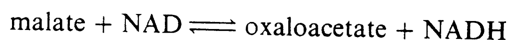


Figure 19-7 Effects of oxaloacetate/NAD concentration on the rate of isotope exchange at equilibrium for the oxaloacetate ↔ malate and the NAD ↔ NADH exchanges. The data for the NAD ↔ NADH exchange are shown at 10 times their appropriate scaled rate. The oxaloacetate/NAD ratio is kept constant as the oxaloacetate concentration is varied.

reactant concentration in increased. In addition, it is self-evident that the maximum rate of all exchanges is the same, since by definition in a rapid-equilibrium, random-order mechanism the interconversion of EAB and EPQ is rate limiting, and all exchanges must proceed through this step.

If we consider the example of malate dehydrogenase, a further useful facet of isotope-exchange studies at equilibrium is illustrated. This enzyme catalyzes the reaction



$$\text{malate} + \text{NAD} \rightleftharpoons \text{oxaloacetate} + \text{NADH}$$

If we examine the effects of raising the concentrations of NAD and oxaloacetate on the rate of NAD $\rightleftharpoons$ NADH exchange, or the rate of oxaloacetate $\rightleftharpoons$ malate exchange (Fig. 19-7), we find that the NAD $\rightleftharpoons$ NADH exchange is inhibited, while the oxaloacetate $\rightleftharpoons$ malate exchange rate reaches a maximum and shows no inhibition.

In addition, the maximum rate of the NAD $\rightleftharpoons$ NADH exchange is *slower* than that of oxaloacetate-malate. These observations are consistent with the ordered addition of NAD and malate, and the ordered release of oxaloacetate and NADH, and furthermore, suggest that NADH release may be the overall rate-limiting step in the reaction. When the concentrations of NADH and malate are raised together, it is found that the rates of *both* exchanges decrease. This suggests that an E-NADH–malate abortive complex is formed: When this occurs the concentrations of *all* the enzyme complexes on the exchange pathways are decreased, leading to an inhibition of *all* exchange rates under conditions that favor formation of the abortive complex (Fig. 19-8).

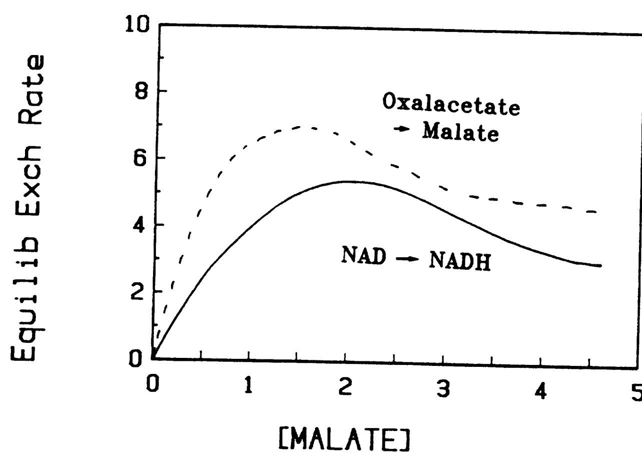


Figure 19-8 Effects of malate/NADH concentration on the rate of isotope exchange at equilibrium for the oxaloacetate ↔ malate and the NAD ↔ NADH exchange. The malate/NADH ratio is kept constant as the malate concentration is raised. Both exchange rates show inhibition as the result of abortive complex formation.

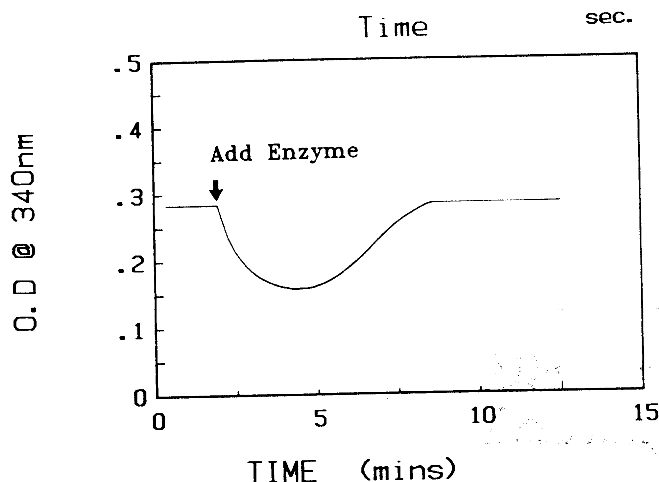


Figure 19-9 Equilibrium mixture established prior to addition of enzyme. NADP + malate-2-D (in place of malate-2-H) + CO_2 + pyruvate + NADPH. Malic enzyme as indicated.

EQUILIBRIUM PERTURBATION STUDIES

Consider an experiment involving the malic enzyme, which catalyzes the reaction

$$\text{NADP} + \text{malate} \rightleftharpoons \text{NADPH} + \text{pyruvate} + CO_2$$

The overall chemical reaction has an equilibrium constant that is first determined. From this the equilibrium concentrations of each reactant can be calculated, and an equilibrium mixture, *in the absence of the enzyme*, established. If this mixture is set up with enzyme added and the optical density at 340 nm (due to the NADPH) monitored, one expects to see no change in OD at 340 nm. If, however, one set up the same equilibrium mixture but replaced the malate with malate deuterated at the 2-position and followed the absorbance at 340 nm after the enzyme had been added; then a distinct change in absorbance is observed, as illustrated in Fig. 19-9.

This perturbation from equilibrium is caused by the more rapid reaction of NADPH + CO_2 + pyruvate to form malate + NADP than the reaction of NADP + malate-2-D to form NADPD + pyruvate + CO_2. In the first part of this discussion the rate of NADP + malate → NADPH + pyruvate + CO_2 (k_1) equals the rate of CO_2 + pyruvate + NADPH → NADP + malate (k_2), as required by the equilibrium condition. In the latter case, with malate-2-D, however, k_1 is less than k_2 because of an isotope effect. As the equilibrium process proceeds, isotopic equilibrium is reached and the system returns to chemical equilibrium. In effect, one is measuring a difference spectrum between two approximately first-order approaches to equilibrium. Apart from providing a simple way of demonstrating an isotope effect, this equilibrium perturbation method has been used to distinguish between compulsory-order and random-order mechanisms.

Figure 19-10 Compulsory-order mechanism.

For particular mechanisms, rate equations for the conversion of, for example, labeled A to P and unlabeled P to A can be derived. In terms of the mechanism shown in Fig. 19-10, the following equations describe these rates.

$$\frac{-dA_D}{dt} = \left(\frac{k_1k_3k_5k_{7D}(E)}{k_2k_4k_6}A_DB - \frac{k_{8D}k_{10}k_{12}(EQ)}{k_9k_{11}}P_D\right) \Bigg/ \left\{1 + \frac{k_{7D}}{k_6}\left[1 + \frac{k_5}{k_4}\left(1 + \frac{k_3B}{k_2}\right)\right] - \frac{k_{9D}}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\right)\right\} \tag{19-4}$$

$$\frac{dA_H}{dt} = \left(\frac{k_{8H}k_{10}k_{12}(EQ)}{k_9k_{11}}P_H - \frac{k_1k_3k_5k_{7H}(E)}{k_2k_4k_6}A_HB\right) \Bigg/ \left\{1 + \frac{k_{7H}}{k_6}\left[1 - \frac{k_5}{k_4}\left(1 - \frac{k_3B}{k_2}\right)\right] - \frac{k_{9H}}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\right)\right\} \tag{19-5}$$

The subscripts H and D refer to either concentrations or rate constants for hydrogen- or deuterium-substituted A or P. Under conditions where the equilibrium levels of B and Q do not change during the perturbation, we can write

$$EQ = E\left(\frac{k_{14}Q}{k_{13}}\right) \tag{19-6}$$

Furthermore, we can define an equilibrium isotope effect

$$\frac{K_{eqH}}{K_{eqD}} = \frac{k_{7H}k_{8D}}{k_{8H}k_{7D}} = \frac{1}{\beta} \tag{19-7}$$

The equation for $-dA/dt$ after rearrangement and substitution for EQ becomes

$$\frac{-dA_D}{dt} = \left(\frac{k_1k_3k_5k_{7H}A_DB}{k_2k_4k_6} - \frac{k_{8H}k_{10}k_{12}k_{14}P_DQ}{\beta k_9k_{11}k_{13}}\right)(E) \Bigg/ \left(\frac{k_{7H}}{k_{7D}} + C_f + \frac{C_r}{\beta}\right) \tag{19-8}$$

and the two phenomenological parameters C_f and C_r are defined by

$$C_f = \frac{k_{7H}}{k_6}\left[1 + \frac{k_5}{k_4}\left(1 + \frac{k_3B}{k_2}\right)\right] \tag{19-9}$$

$$C_r = \frac{k_{9H}}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\right) \tag{19-10}$$

In the context of distinguishing ordered from random mechanisms, we need only consider the parameter C_f, which, for the compulsory ordered mechanism shown earlier, has a linear dependence on the concentration of the second substrate, B. C_f

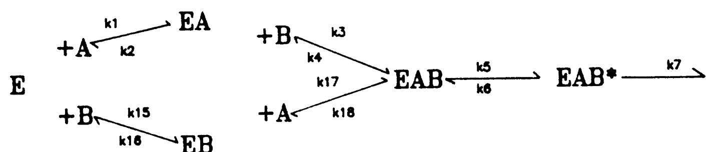


Figure 19-11 Random-order mechanism.

reflects the commitment of the ternary EAB complex to catalysis. In a compulsory-order mechanism with A as the leading substrate, C_f is infinite at an infinite concentration of B. If, however, A can appreciably dissociate from EAB (i.e., the mechanism has some component of randomness). C_f is not infinite at an infinite concentration of B. For a random addition of A and B, as shown in Fig. 19-11, the expression for C_f is given by

$$C_f = \frac{k_{7H}}{k_6}\left(1 + \frac{k_5}{k_{10}}\right) \tag{19-11}$$

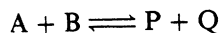
which, at infinite concentrations of B, becomes

$$C_f = \frac{k_{7H}}{k_6}\left[1 + \frac{k_5}{k_4/(1 + k_3\mathrm{B}/k_2) + k_{10}}\right] \tag{19-12}$$

Values of C_f (and C_r) are obtained from perturbation experiments by solving these (and other derived) equations, usually by computer, but this requires a knowledge of the equilibrium isotope effect. Equilibrium isotope effects are determined by careful determination of the equilibrium constant with protonated or deuterated compounds.

MEASUREMENT OF FLUX RATIOS

One of the problems that many approaches available for determining the formal kinetic mechanism of an enzyme-catalyzed reaction have is the fact that any complexity, such as abortive complex formation or allosteric interactions and enzyme isomerizations, can interfere with the interpretation of initial rate studies, inhibition studies, or even isotope exchange at equilibrium studies. One approach circumvents most of these difficulties by isolating the *order* of substrate binding from other aspects of the mechanism. This is the so-called *flux ratio* method. In many respects it resembles a product inhibition technique, but rather than examining effects on initial rates, it examines the fate of individual product molecules participating in inhibitory reactions. The approach is best understood by examining what one would expect in an experiment involving a two-substrate reaction:



$$\mathrm{A} + \mathrm{B} \rightleftharpoons \mathrm{P} + \mathrm{Q}$$

Using a suitably labeled product, P, one can measure *two* fluxes, one involving $\mathrm{P} \rightarrow \mathrm{A}$ and the other $\mathrm{P} \rightarrow \mathrm{B}$. If we consider the mechanism shown in Fig. 19-12, equations for these fluxes can be derived, but the expressions would be extremely

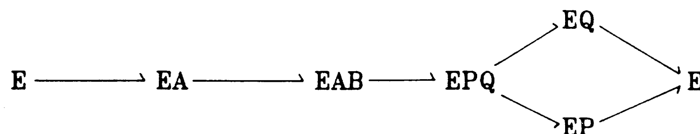


Figure 19-12 Mechanism involving a compulsory order of substrate addition and a random order of product release.

complex. However, as we will see, the useful parameter is the *ratio* between the fluxes for P → A and P → B.

For this mechanism, consider the *fate* of a molecule of P that traps a molecule of EQ produced as an intermediate in the breakdown of EAB. Once P has bound to EQ, we get formation of EPQ, which can do one of many things, as outlined in Fig. 19-13.

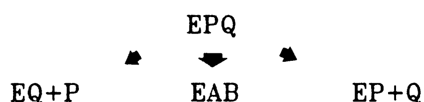


Figure 19-13 Fates of EPQ molecule possible in the mechanism shown in Fig. 19-12.

Obviously only EPQ molecules following the route to EAB can contribute to the flux of P → A. Once EAB is formed various routes can be taken. EAB can be converted to EA + B or back to EPQ and, as before, only EA can contribute to the flux of P → A. EA, once formed, can dissociate to E and A or bind B and return to EAB. Similar considerations contribute to the flux of P → B, and it is quite easy to see why expressions for these two fluxes are complex.

However, if one examines the pathways for P → A and for P → B, they are essentially similar; the formation of A requires the extra step EA → E + A. As a result, the flux ratio [Eq. (19-13)] is determined essentially by the immediate fate of EA formed by back reaction from P′.

$$\text{flux ratio} = \frac{F_{P \to B}}{F_{P \to A}} \tag{19-13}$$

The *fate* of this molecule of EA depends on the *concentration* of B but not A. At low concentrations of B, EA appreciably dissociates to give E + A (i.e., flux of P → A occurs). At high concentrations of B, EA is trapped back into EAB and flux does not take place. Thus we can write

$$\frac{F_{P \to B}}{F_{P \to A}} = 1 + \alpha[B] \tag{19-14}$$

where α is a constant. Figure 19-14 illustrates the dependence of the flux ratio for this compulsory order of addition of A and B on the concentrations of A and B.

If there is a *random* order of addition of A and B to give a ternary complex, a more complex situation holds, and nonlinear dependence of the flux ratio on the

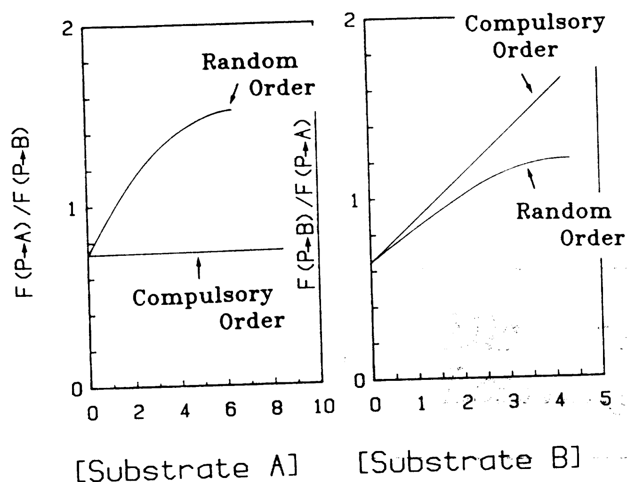


Figure 19-14 Expected patterns for flux-ratio measurements for random-order mechanisms (----) and for compulsory-order mechanisms (——).

concentration of either A or B results, as shown by the dashed lines in Fig. 19-14.

To examine the order of product release (i.e., the order of substrate addition in the reverse reaction), flux ratios for A → P and A → Q must be examined as a function of the concentrations of P and Q.

SUBSTRATE "STICKINESS"

In considering *random-order* mechanisms it is possible to determine when a mechanism is rapid equilibrium as opposed to steady state. In rapid-equilibrium order, one is basically saying that the interconversion of EAB → EPQ is a much slower step than EA + B ⇌ EAB or EB + A ⇌ EAB. This assumption can be directly tested experimentally using isotopically labeled substrates.

Consider the following experiment. Enzyme is premixed with isotopically labeled A (A*) to give EA*. This EA* is then added to a large excess of A and B and the amount of A* transferred into product is measured.

If the mechanism is true rapid equilibrium, random order, the *specific activity* of the product should approach that of A* *after* the addition of the excess A. This is so because each preformed molecule of EA* dissociates far more rapidly than it proceeds to product, allowing isotopic equilibration before significant product formation occurs; therefore, product and A* (after mixing) have similar specific activities. If, on the other hand, one approaches the situation where $k_{cat} = k_8$ in the scheme in Fig. 19-15, the specific activity of the product formed in the experiment above is much higher than in the true rapid-equilibrium, random-order case. Similarly, if one premixes E with B* to give EB* and follows a similar procedure, the relative magnitudes of k_{cat} and k_6 in the Fig. 19-15 scheme can be determined.

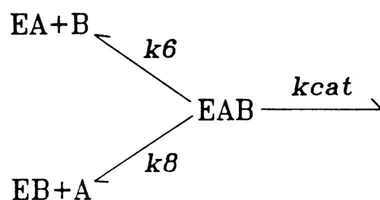


Figure 19-15 Scheme of a random-order mechanism.

This type of experiment is extremely useful in determining the "degree of randomness" of a random-order mechanism, or in other words, how closely the mechanism approaches the true rapid-equilibrium, random-order situation. If it does not obey rapid equilibrium kinetics, relationships giving dissociation constants for binary EB complexes and the equality of Michaelis constants with dissociation constants of the appropriate ternary complexes do not hold, and the mechanism can exist as a kinetically preferred pathway of substrate addition.

ELUCIDATION OF RATE DETERMINING STEPS

For an enzyme having a random-order addition of substrates that does obey the rapid-equilibrium condition, k_{cat} is the overall rate-limiting step. It is usually written as EAB → EPQ, implying that this rate constant is that for the chemical reaction. Although this may well be the case, the rapid-equilibrium condition requires only that some step (other than substrate addition or product release) involving the central complexes be rate limiting. This step could equally well be a rate-limiting conformational change involving EAB or EPQ required to allow catalysis or product release, as summarized in Fig. 19-16.

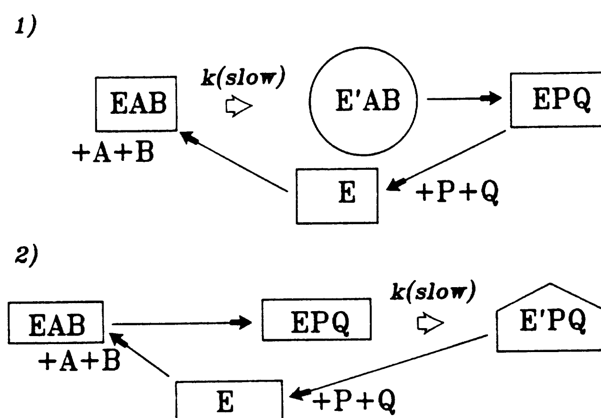


Figure 19-16 Schemes of ternary complex mechanisms involving rate-limiting conformational changes.

In such a case, studies of isotope effects on the V_{max} parameter (θ_0) allows a distinction to be made. For a rapid-equilibrium, random-order mechanism, $\theta_0 = 1/k_{cat}$, and if k_{cat} is the *chemical* step in the interconversion of EAB → EPQ, use of a substrate with an isotope at the bond-breaking atom gives a primary isotope effect, detected by initial rate studies as a change in θ_0. If, on the other hand, a rate-determining conformational change is occurring, no isotope effect on θ_0 is expected. In the case of a mechanism involving a hydride transfer step (e.g., a dehydrogenase), substitution with tritium results in a large primary isotope effect (in the range 3- to 10-fold) if the hydride transfer step is, in fact, rate limiting. The magnitude of the effect is important, as the isotopic substitution could make the hydride transfer step become rate limiting even though some other step is, with the normal substrate, slower. Under this circumstance, the magnitude of the effect is less than that expected for a primary isotope effect.

For non-rapid-equilibrium, random-order mechanisms, isotope effects on individual θ parameters are usually smaller than the effects observed on θ_0 in a rapid-equilibrium, random-order mechanism. This is so because in general the k_{cat} term appears, together with other rate constants, in a number of the θ parameters. For a compulsory-order mechanism only the θ_1 parameter does *not* contain k_{cat} and as a result is immune to an isotope effect. The other parameters show an isotope effect, but because of a "buffering effect" of rate constants not involved in the chemical interconversion, they have a less-than-maximal effect.

Stopped-flow studies in these cases allow examination for the existence of a primary isotope effect. In a compulsory-order mechanism, with rate-limiting product release, a pre-steady-state "burst" is observed and its rate includes the chemical interconversion step (strictly speaking, the k_{cat} step, as in the previous discussion for rapid-equilibrium, random-order mechanisms). This allows one to look for an isotope effect in the "burst" phase of the reaction.

We have considered isotope effects here as involving substitution of a proton by tritium or deuterium, cases that clearly optimize the magnitude of an expected isotope effect. In steady-state studies it is not unreasonable to expect to be able to experimentally determine *primary* isotope effects associated with bond-breaking steps involving carbon or nitrogen atoms, although such effects would be difficult to establish in pre-steady-state studies.

Isotopically labeled substrates provide a variety of means for obtaining information on the kinetic mechanism of an enzyme-catalyzed reaction. Apart from experiments involving substrate "stickiness" and isotope effects, much knowledge can also be gained in principle from the kinetic techniques discussed in Chaps. 13 to 15. Isotopically labeled substrates are invaluable where the approaches described in those chapters cannot readily be employed. As stated in the last two sections of this chapter, the use of isotopically labeled substrates can provide a wealth of information that is not available from initial rate kinetic studies.