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THE USE OF COMPETITIVE INHIBITORS IN STUDYING THE MECHANISM OF ACTION OF SOME ENZYME SYSTEMS UTILIZING THREE SUBSTRATES

HERBERT J. FROMM
Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa (U.S.A.)

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SUMMARY

A kinetic approach is presented for studying the mechanism of action of enzyme systems which utilize three substrates. Such systems may be segregated into two classifications based upon their initial rate expressions. One class of mechanisms is made up of sequential pathways of enzyme and substrate interaction while the second is of the "ping-pong" type. It is possible to further separate the mechanisms in each category either from initial rate experiments alone, or from studies with competitive inhibitors. It is possible, by using competitive inhibitors in kinetic experiments, to obtain information on the binding order of substrates to enzymes for many ordered systems. This analysis has the advantage that in most cases definitive information on the mechanism of enzyme action can be obtained from initial velocity studies in a single direction only.

INTRODUCTION

Kinetic studies of enzyme reactions have been limited essentially to systems involving one and two substrates. Only in a few isolated reports have investigators directed their attention to initial rate studies of three substrate enzymic systems[1-3]. It would appear at first glance, based upon the obvious kinetic complexity of two substrate systems, that little fruitful information could be obtained conveniently from initial rate experiments with enzymes which utilize three substrates. However, as will be shown below, information on the mechanism of action of such systems can readily be obtained from initial rate experiments in which kinetic investigations are carried out in the absence and presence of substrate analogs which compete with substrates for a similar site on the enzyme.

In 1964 it was shown how competitive inhibitors of substrates or weak alternative substrates might be employed to permit a choice to be made between "ordered" and "random" two substrate systems[4]. This type of kinetic approach was used earlier with yeast hexokinase (EC 2.7.1.1)[5,6]. Another advantage of using compounds which

compete with substrates for the same enzymic site in ordered systems is that the sequence of substrate addition can also be determined[4].

It is possible by using competitive inhibitors and weak alternative substrates to make a definitive choice of mechanism in the case of three substrate systems. As will be shown below, it is possible to segregate three substrate systems into two classes based upon initial rate equations alone. One class is of the so-called "ping-pong" type in which a substrate reacts with the enzyme to produce a product before the addition of a different substrate species. The other class of systems require that all three substrates be present on the enzyme prior to product formation.

Experimentally, it is necessary to vary one substrate while holding the other two reactants at a fixed level in the general concentration range of their Michaelis constants. This experiment is then repeated but at a different concentration of fixed substrates, care being exercised to maintain the ratio of fixed substrates constant in the two studies. When this experimental attack is used, it will be observed that LINEWEAVER–BURK[7] plots for those mechanisms involving quarternary complexes will yield lines that converge at the left of the 1/velocity axis. In the case of ping-pong mechanisms one or more sets of data will give parallel plots.

After segregation of the two classes of mechanisms, additional investigations, in which competitive inhibitors of substrates are employed, may be used. In the schemes of enzyme and substrate interactions listed below it should be borne in mind that, although many of the listed mechanisms have not as yet been found to be applicable to actual enzyme systems, they are in fact analogous to mechanisms for two substrate systems and are thus not completely hypothetical.

RATE EQUATIONS

The mechanisms of enzyme and substrate interaction are listed below under two classifications. Under CASE I are shown initial rate equations which yield converging Lineweaver–Burk type plots when kinetic experiments are carried out as described above. The mechanisms listed under CASE II give initial rate equations in which parallel lines are obtained in one or more double reciprocal plot. When the inhibition studies are carried out it will be necessary to vary one substrate while holding the other two substrates constant. Under these conditions velocities will be determined in the presence and absence of inhibitor.

CASE I

A. Ordered mechanism of enzyme and substrate interaction leading to a single central quarternary complex. The rate equation for this mechanism was first derived by FRIEDEN[1]. Using the nomenclature of CLELAND[2]. the mechanism can be written as follows:

A	B	C	P	Q	R
$k_1 \downarrow k_2$	$k_3 \downarrow k_4$	$k_5 \downarrow k_6$	$k_7 \uparrow k_8$	$k_9 \uparrow k_{10}$	$k_{11} \uparrow k_{12}$

E EA EAB EXYZ EQR ER E

Scheme I

The steady state rate equation for this mechanism when transposed into a form suggested by DALZIEL[8] is

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A} + \frac{\Phi_2}{B} + \frac{\Phi_3}{C} + \frac{\Phi_4}{(A)\,(B)} + \frac{\Phi_5}{(B)\,(C)} + \frac{\Phi_6}{(A)\,(B)\,(C)}, \tag{1a}$$

where E_0 represents total enzyme concentration and v, initial velocity. A, B, and C represent substrates as depicted in Scheme I, and R, Q and P are taken to be their respective products. The various Φ parameters which are combinations of rate constants are:

$$\Phi_0 = \frac{1}{k_7} + \frac{1}{k_9} + \frac{1}{k_{11}}\,;\ \Phi_1 = \frac{1}{k_1}\,;\ \Phi_2 = \frac{1}{k_3}\,;\ \Phi_3 = \frac{(k_6 + k_7)}{k_5 k_7}$$

$$\Phi_4 = \frac{k_2}{k_1 k_3}\,;\ \Phi_5 = \frac{k_4(k_6 + k_7)}{k_3 k_5 k_7}\,;\ \Phi_6 = \frac{k_2 k_4 (k_6 + k_7)}{k_1 k_3 k_5 k_7}$$

When a compound is present which competes with the substrate for the same site on the enzyme the following interactions and rate equations might be expected.

1. Inhibitor for A—Inhibitor, I, competes with A for same enzymatic site.

$$E + I = EI,\ K_1;\ EI + B = EIB,\ K_2;\ EIB + C = EIBC,\ K_3$$

$$\frac{E_0}{v} = \Phi_0 + \frac{1}{A}\left(\Phi_1 + \frac{\Phi_1(I)}{K_1} + \frac{\Phi_1(I)\,(B)}{K_1K_2} + \frac{\Phi_1(I)\,(B)\,(C)}{K_1K_2K_3} + \frac{\Phi_4(I)}{K_1K_2} + \right. \tag{1b}$$

$$\left. \frac{\Phi_4(I)\,(C)}{K_1K_2K_3} + \frac{\Phi_6(I)}{K_1K_2K_3}\right) +$$

$$\frac{\Phi_2}{B} + \frac{\Phi_3}{C} + \frac{\Phi_4}{(A)\,(B)}\left(1 + \frac{I}{K_1}\right) + \frac{\Phi_5}{(B)\,(C)} + \frac{\Phi_6(I)}{K_1K_2(A)\,(C)} + \frac{\Phi_6}{(A)\,(B)\,(C)}\left(1 + \frac{I}{K_1}\right)$$

Eqn. 1b predicts that inhibition with respect to substrate A will be competitive; however, this same inhibitor will give rise to non-linear double reciprocal plots when either B or C is the varied substrate.

2. Inhibitor for B—Inhibitor I, competes with B for same enzymatic site. The likely interactions are:

$$EA + I = EAI,\ K_1;\ EAI + C = EAIC,\ K_2$$

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A} + \frac{1}{B}\left(\Phi_2 + \frac{\Phi_2(I)}{K_1} + \frac{\Phi_2(I)\,(C)}{K_1K_2} + \frac{\Phi_5(I)}{K_1K_2}\right) + \frac{\Phi_3}{C} \tag{1c}$$

$$+ \frac{\Phi_4}{(A)\,(B)} + \frac{\Phi_5}{(B)\,(C)}\left(1 + \frac{I}{K_1}\right) + \frac{\Phi_6}{(A)\,(B)\,(C)}$$

Eqn. 1c predicts that inhibition with respect to substrate A will be uncompetitive while inhibition relative to B will be of the competitive type; however, inspection of Lineweaver–Burk plots where C is varied at a fixed level of A and B will indicate non-linear inhibition.

3. Inhibitor for C—Inhibitor I competes with C for same enzymatic site.

$$EAB + I = EABI,\ K_1$$

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A} + \frac{\Phi_2}{B} + \frac{\Phi_3}{C}\left(1 + \frac{I}{K_1}\right) + \frac{\Phi_4}{(A)\,(B)} + \frac{\Phi_5}{(B)\,(C)} + \frac{\Phi_6}{(A)\,(B)\,(C)} \tag{1d}$$

It can be seen from Eqn. 1d that the inhibition patterns relative to substrates A and B are uncompetitive while inhibition with respect to C is competitive.

It is apparent from the rate equations listed above that the inhibition patterns differ for each substrate inhibitor. The uniqueness of these inhibitory effects relative to each substrate permits one to establish the order of addition of substrates to the enzyme.

For certain enzyme systems exhibiting the mechanism depicted above, the presence of the inhibitor on the enzyme will prevent addition of other substrate molecules to the enzyme, *i.e.*, if a competitive inhibitor for A forms complex EI, then EIB and EIBC will not occur. Under these limiting conditions terms in Eqns. 1b and 1c containing K_2 and K_3 will be deleted. It will still be possible, however, to determine the binding order of substrates to enzyme under these limiting conditions.

B. Rapid-equilibrium random mechanism. This mechanism is similar to that suggested by ALBERTY[9] in 1953 for two substrate systems. The initial rate equation (1e) was first derived by FRIEDEN[1] in 1959. For the mechanism depicted in Scheme II, it is assumed that all steps in the sequence equilibrate rapidly relative to the interconversions of the quarternary complexes. For the sake of brevity all those equilibria which occur after the formation of complex EPQR are deleted from Scheme II.

$$\begin{array}{lll} E + A = EA, \quad K_1 & EC + B = EBC, \quad K_5 & EB + C = EBC, \quad K_9 \\ E + B = EB, \quad K_2 & EB + A = EAB, \quad K_6 & EAB + C = EABC, \quad K_{10} \\ E + C = EC, \quad K_3 & EC + A = EAC, \quad K_7 & EAC + B = EABC, \quad K_{11} \\ EA + B = EAB, \quad K_4 & EA + C \underset{k_1}{=} EAC, \quad K_8 & EBC + A = EABC, \quad K_{12} \end{array}$$

$$EABC \underset{k_2}{\rightleftharpoons} EPQR$$

Scheme II

The rate equation for the mechanism depicted in Scheme II is

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A} + \frac{\Phi_2}{B} + \frac{\Phi_3}{C} + \frac{\Phi_4}{(A)\,(C)} + \frac{\Phi_5}{(A)\,(B)} + \frac{\Phi_6}{(B)\,(C)} + \frac{\Phi_7}{(A)\,(B)\,(C)} \tag{1e}$$

The various Φ values are,

$$\Phi_0 = \frac{1}{k_1},\ \Phi_1 = \frac{K_{12}}{k_1},\ \Phi_2 = \frac{K_{11}}{k_1},\ \Phi_3 = \frac{K_{10}}{k_1},\ \Phi_4 = \frac{K_9 K_{12}}{k_1},\ \Phi_5 = \frac{K_7 K_{11}}{k_1},\ \Phi_6 = \frac{K_8 K_{11}}{k_1},$$

$$\Phi_7 = \frac{K_1 K_8 K_{11}}{k_1}$$

If an inhibitor (I) is used which competes with A for the same site on the enzyme, the following equilibria are to be expected:

$$\begin{array}{lll} E + I = EI, \quad K_1' & EI + C = EIC, \quad K_4' & EIB + C = EIBC, \quad K_7' \\ EI + B = EIB, \quad K_2' & EIC + B = EIBC, \quad K_5' & EC + I = EIC, \quad K_8' \\ EIB + C = EIBC, \quad K_3' & EB + I = EIB, \quad K_6' & EBC + I = EIBC, \quad K_9' \end{array}$$

Scheme III

The rate equation for the effect of inhibition is

$$\frac{E_0}{v} = \Phi_0 + \frac{1}{A}\left(\Phi_1 + \frac{\Phi_7(I)}{K_1'K_2'K_3'}\right) + \frac{\Phi_2}{B} + \frac{\Phi_3}{C} + \frac{1}{(A)\,(C)}\left(\Phi_4 + \frac{\Phi_7(I)}{K_1'K_2'}\right) + \tag{1f}$$

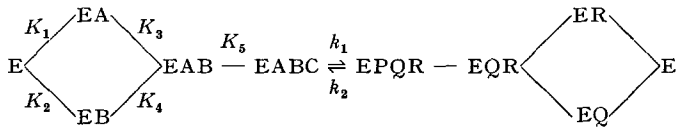
$$+ \frac{1}{(A)\,(B)}\left(\Phi_5 + \frac{\Phi_7(I)}{K_1'K_4'}\right) + \frac{\Phi_6}{(B)\,(C)} + \frac{\Phi_7}{(A)\,(B)\,(C)}\left(1 + \frac{I}{K_1'}\right)$$

It is apparent from Eqn. 1f that inhibition relative to substrate A will be com-

petitive; however, it will be mixed relative to the other substrates. A similar type of inhibition pattern will be observed when inhibitors of B and C are substituted for A, *i.e.*, a competitive inhibitor for B will exhibit mixed inhibition patterns for substrates A and C.

C. There are two limiting cases of the rapid-equilibrium random mechanism discussed above. These are partial random mechanisms and can readily be differentiated from each other and all other mechanisms to be presented in this report on the basis of their initial rate equations with and without competitive inhibitors.

1. *Random addition of substrates A and B*—In Scheme IV is shown the first of these two partial random mechanisms.



Scheme IV

If all steps except the interconversion of the quarternary complexes equilibrate rapidly, the rate expression for the mechanism described in Scheme IV is

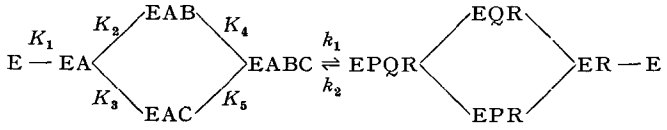
$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{C} + \frac{\Phi_2}{(A)\,(C)} + \frac{\Phi_3}{(B)\,(C)} + \frac{\Phi_4}{(A)\,(B)\,(C)} \tag{1g}$$

where

$$\Phi_0 = \frac{1}{k_1},\ \Phi_1 = \frac{K_5}{k_1},\ \Phi_2 = \frac{K_4K_5}{k_1},\ \Phi_3 = \frac{K_3K_5}{k_1} \text{ and } \Phi_4 = \frac{K_1K_3K_5}{k_1}$$

It can be observed from Eqn. 1g that when C is the varied substrate at fixed but different levels of A and B, where the ratio of A and B are maintained constant, the Lineweaver–Burk plots will converge at a common point on the 1/velocity axis. On the other hand, when either A or B are the varied substrates, the plots will meet to the left of the 1/velocity axis. It will thus be possible simply to determine which substrates add randomly to the enzyme.

2. *Random addition of substrates B and C*—If substrate A is the obligatory first substrate to add to the enzyme and if substrates B and C can then add in random fashion, the mechanism is depicted as follows.



Scheme V

The rate equation for Scheme V, making the usual assumptions as stated above is

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{B} + \frac{\Phi_2}{C} + \frac{\Phi_3}{(B)\,(C)} + \frac{\Phi_4}{(A)\,(B)\,(C)} \tag{1h}$$

where

$$\Phi_0 = \frac{1}{k_1},\ \Phi_1 = \frac{K_5}{k_1},\ \Phi_2 = \frac{K_4}{k_1},\ \Phi_3 = \frac{K_2K_4}{k_1},\ \Phi_4 = \frac{K_1K_2K_4}{k_1}$$

Although it is not possible to differentiate between Equations 1a, 1e, and 1h from initial rate experiments alone, a choice between the three mechanisms yielding these rate equations can be made by using competitive inhibitors.

1. Competitive inhibitor for A—A competitive inhibitor for A can react with the various enzyme forms as follows: E + I = EI, K_1; EI + B = EIB, K_2; EI + C = EIC, K_3; EIB + C = EIBC, K_4; EIC + B = EIBC, K_5

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_4(I)}{K_1K_2K_4(A)} + \frac{\Phi_1}{B} + \frac{\Phi_2}{C} + \frac{\Phi_4(I)}{K_1K_3(A)\ (B)} + \frac{\Phi_4(I)}{K_1K_2(A)\ (C)} + \frac{\Phi_3}{(B)\ (C)} + \frac{\Phi_4}{(A)\ (B)\ (C)}\left(1 + \frac{I}{K_1}\right) \tag{1i}$$

Inhibition, although competitive relative to substrate A will be mixed with respect to the other substrates.

2. Competitive inhibitor for B—A competitive inhibitor for B can be expected to react in this system as follows:

EA + I = EAI, K_1; EAI + C = EAIC, K_2; EAC + I = EAIC, K_3

$$\frac{E_0}{v} = \Phi_0 + \frac{1}{B}\left[\Phi_1 + \frac{\Phi_3(I)}{K_1K_2}\right] + \frac{\Phi_2}{C} + \frac{\Phi_3}{(B)\ (C)}\left[1 + \frac{I}{K_1}\right] + \frac{\Phi_4}{(A)\ (B)\ (C)} \tag{1j}$$

Equation 1j indicates that for this mechanism, a competitive inhibitor for B gives uncompetitive inhibition with respect to A and mixed inhibition relative to C.

Because substrates B and C may add randomly, the type of inhibition pattern to be expected when a competitive inhibitor for C is used, will be similar to that shown above. Thus a competitive inhibitor for C exhibits uncompetitive and mixed inhibition relative to substrates A and B. It is therefore possible to identify substrate A for this mechanism.

It should be noted that the inhibition patterns relative to the mechanisms of Schemes I, II, and V are different, and thus a choice of mechanism can be made between these possible pathways.

A third type of partially random mechanism of substrate addition to the enzyme is possible for three substrate systems. For this case, B is the obligatory second substrate while either A or C may add to the enzyme randomly. If the assumptions outlined above for random mechanisms are made, it can be shown that for this mechanism the initial rate equation contains denominator terms in A, C, (A) (B), (B) (C), and (A) (B) (C). The inhibition patterns are similar, when competitive inhibitors are employed, to those described for Scheme II, and thus a choice cannot be made between these two pathways of enzyme and substrate interaction.

CASE II. PING-PONG MECHANISMS (CLELAND[2])

There are three ping-pong type mechanisms for three substrate systems only one of which has a unique initial rate equation. One cardinal feature of these mechanisms is that when a substrate is varied at fixed but different levels of the other two substrates, but where these latter two reactions are maintained in a constant ratio, one or more of the Lineweaver–Burk plots will yield parallel lines.

A. Ping-pong mechanism 1. The sequence of enzyme and substrate interaction is as follows:

$$\begin{array}{ccccccccccccc} & & \mathrm{A} & & \mathrm{P} & & \mathrm{B} & & \mathrm{Q} & & \mathrm{C} & & \mathrm{R} \\ & & k_1 \downarrow k_2 & & k_3 \uparrow k_4 & & k_5 \downarrow k_6 & & k_7 \uparrow k_8 & & k_9 \downarrow k_{10} & & k_{11} \uparrow k_{12} \\ \mathrm{E} & & \begin{pmatrix}\mathrm{EA}\\ \mathrm{EP}\end{pmatrix} & & \mathrm{F} & & \begin{pmatrix}\mathrm{FB}\\ \mathrm{GQ}\end{pmatrix} & & \mathrm{G} & & \begin{pmatrix}\mathrm{GC}\\ \mathrm{ER}\end{pmatrix} & & \mathrm{E} \end{array}$$

Scheme VI

The velocity expression for this mechanism is

$$\frac{\mathrm{E}_0}{v} = \Phi_0 + \frac{\Phi_1}{\mathrm{A}} + \frac{\Phi_2}{\mathrm{B}} + \frac{\Phi_3}{\mathrm{C}} \tag{2a}$$

where

$$\Phi_0 = \frac{1}{k_3} + \frac{1}{k_7} + \frac{1}{k_{11}},\ \Phi_1 = \frac{(k_2 + k_3)}{k_1 k_3},\ \Phi_2 = \frac{(k_6 + k_7)}{k_5 k_7} \text{ and } \Phi_3 = \frac{(k_{10} + k_{11})}{k_9 k_{11}}$$

It is apparent from Eqn. 2a that Lineweaver–Burk plots when made using the experimental protocol outlined above give parallel lines. This is the only mechanism, of those to be considered in this report that yields kinetic data of this type.

B. Ping-pong mechanism 2. Scheme VII shows a second type of ping-pong mechanism for three substrate systems.

$$\begin{array}{ccccccccccccc} & & \mathrm{A} & & \mathrm{B} & & \mathrm{P} & & \mathrm{C} & & \mathrm{Q} & & \mathrm{R} \\ \mathrm{E} & & k_1 \downarrow k_2 & & k_3 \downarrow k_4 & & k_5 \uparrow k_6 & & k_7 \downarrow k_8 & & k_9 \uparrow k_{10} & & k_{11} \uparrow k_{12} & & \mathrm{E} \\ & & \mathrm{EA} & & \mathrm{EX} & & \mathrm{EY} & & \mathrm{EZ} & & \mathrm{ER} & & \end{array}$$

Scheme VII

$$\frac{\mathrm{E}_0}{v} = \Phi_0 + \frac{\Phi_1}{\mathrm{A}} + \frac{\Phi_2}{\mathrm{B}} + \frac{\Phi_3}{\mathrm{C}} + \frac{\Phi_4}{(\mathrm{A})\,(\mathrm{B})}, \tag{2b}$$

where

$$\Phi_0 = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_1},\ \Phi_1 = \frac{1}{k_1},\ \Phi_2 = \frac{(k_4 + k_5)}{k_3 k_5},\ \Phi_3 = \frac{(k_8 + k_9)}{k_7 k_9} \text{ and } \Phi_4 = \frac{k_2(k_4 + k_5)}{k_1 k_3 k_5}$$

It is apparent from Eqn. 2b that Lineweaver–Burk plots give linear parallel curves where C is the varied substrate with A and B held at different levels, but at a fixed ratio. On the other hand when either A or B are the varied substrates, the lines will converge at a common point to the left of the 1/velocity axis. It will be shown below that another type of ping-pong mechanism, mechanism 3, gives a similar initial rate equation.

1. Inhibitor for A—An inhibitor I which competes with A for the same enzymic site might be expected to react as follows: $\mathrm{E} + \mathrm{I} = \mathrm{EI},\ K_1$

$$\frac{\mathrm{E}_0}{v} = \Phi_0 + \frac{1}{\mathrm{A}}\left(\Phi_1 + \frac{\Phi_1(\mathrm{I})}{K_1}\right) + \frac{\Phi_2}{\mathrm{B}} + \frac{\Phi_3}{\mathrm{C}} + \frac{\Phi_4}{(\mathrm{A})\,(\mathrm{B})}\left(1 + \frac{\mathrm{I}}{K_1}\right) \tag{2c}$$

Eqn. 2c suggests that inhibition relative to A, B, and C will be competitive, mixed, and uncompetitive, respectively.

2. Inhibitor for B—A competing inhibitor for B might be expected to interact with the enzyme thusly. $\mathrm{EA} + \mathrm{I} = \mathrm{EAI},\ K_1$

$$\frac{\mathrm{E}_0}{v} = \Phi_0 + \frac{\Phi_1}{\mathrm{A}} + \frac{\Phi_2}{\mathrm{B}}\left(1 + \frac{\mathrm{I}}{K_1}\right) + \frac{\Phi_3}{\mathrm{C}} + \frac{\Phi_4}{(\mathrm{A})\,(\mathrm{B})} \tag{2d}$$

It can be seen from Eqn. 2d that inhibition with respect to substrates A, B, and C is uncompetitive, competitive, and uncompetitive, respectively.

3. Inhibitor for C—An inhibitor of C would be expected to react in a manner analogous to that shown in B2. Thus the interaction would be

$$EY + I = EYI,\ K_I$$

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A} + \frac{\Phi_2}{B} + \frac{\Phi_3}{C}\left(1 + \frac{I}{K_I}\right) + \frac{\Phi_4}{(A)\,(B)} \tag{2e}$$

Inhibition patterns will be uncompetitive relative to substrates A and B and competitive with respect to C.

It will be shown that the rate equations for mechanism 2 differ from those described for other ping-pong types and thus a choice of mechanism can be made. It is also possible to establish the binding order of substrates to enzyme by employing this approach .

It is important when considering this and other ping-pong mechanisms to employ competitive inhibitors of substrates which when present on the enzyme do not form additional complexes with other substrates. In the case of ping-pong mechanism 2, it is possible that EI once formed can react with B to give EIB. Under these conditions inhibition relative to A will be competitive; however, inhibition with respect to B will be non-linear. This can be avoided by selecting inhibitors which give only usual inhibition patterns, *i.e.*, competitive, non-competitive, mixed, and uncompetitive.

C. Ping-pong mechanism 3. In Scheme VIII is shown another type of ping-pong mechanism which gives an initial rate equation similar to one presented above, 2b.

$$\begin{array}{ccccccccccccc} & & A & & P & & B & & C & & Q & & R & \\ & & k_1\downarrow k_2 & & k_3\uparrow k_4 & & k_5\downarrow k_6 & & k_7\downarrow k_8 & & k_9\uparrow k_{10} & & k_{11}\uparrow k_{12} & \\ E & & & EA & & EX & & EB & & EC & & ER & & E \end{array}$$

Scheme VIII

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A} + \frac{\Phi_2}{B} + \frac{\Phi_3}{C} + \frac{\Phi_4}{(B)\,(C)} \tag{2f}$$

where

$$\Phi_0 = \frac{1}{k_3} + \frac{1}{k_9} + \frac{1}{k_{11}},\ \Phi_1 = \frac{(k_2 + k_3)}{k_1 k_3},\ \Phi_2 = \frac{1}{k_5},\ \Phi_3 = \frac{(k_8 + k_9)}{k_7 k_9} \text{ and } \Phi_4 = \frac{k_6(k_8 + k_9)}{k_5 k_7 k_9}$$

For the reverse reaction,

$$\frac{E_0}{v} = \Phi_0' + \frac{\Phi_1'}{R} + \frac{\Phi_2'}{Q} + \frac{\Phi_3'}{P} + \frac{\Phi_4'}{(Q)\,(R)} \tag{2g}$$

where

$$\Phi_0' = \frac{1}{k_2} + \frac{1}{k_6} + \frac{1}{k_8},\ \Phi_1' = \frac{1}{k_{12}},\ \Phi_2' = \frac{(k_8 + k_9)}{k_8 k_{10}}$$

$$\Phi_3' = \frac{(k_2 + k_3)}{k_2 k_4} \text{ and } \Phi_4' = \frac{k_{11}(k_8 + k_9)}{k_8 k_{10} k_{12}}$$

When Lineweaver–Burk plots are made from initial velocity experiments according to the procedure outlined above, one set of results from the forward and reverse reactions will show parallel lines. For the forward reaction, this relationship

will hold when A is the varied substrate, while in the reverse reaction a similar result will be obtained when P is varied at fixed concentrations of Q and R; the latter substrates being maintained at a constant ratio. Because Scheme VIII represents a mechanism which is unsymmetrical, competitive inhibitors of A and R will give different inhibition patterns relative to the other substrates.

Eqn. 2h illustrates the effect of a competitive inhibitor for A on the pathway shown in Scheme VIII while Eqn. 2i shows the effect of a competitive inhibitor for R on this mechanism where complex EIQ is not formed.

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_1}{A}\left(1 + \frac{I}{K_1}\right) + \frac{\Phi_2}{B} + \frac{\Phi_3}{C} + \frac{\Phi_4}{(B)\,(C)}, \tag{2h}$$

where K_1 represents the dissociation constant of the EI complex.

$$\frac{E_0}{v} = \Phi_0' + \frac{\Phi_1'}{R}\left(1 + \frac{I}{K_1}\right) + \frac{\Phi_2'}{Q} + \frac{\Phi_3'}{P} + \frac{\Phi_4'}{(Q)\,(R)}\left(1 + \frac{I}{K_1}\right) \tag{2i}$$

It is apparent that a choice can readily be made between this mechanism and other ping-pong types already considered. By employing competitive inhibitors for substrates B, C, P, and Q the substrate binding order may also be established.

DISCUSSION

In the present paper a kinetic approach was set forth which may be of some value in studying the mechanism of action of three substrate enzyme systems. Certainly, other methods of investigation, such as initial rate studies with product inhibitors[2,10,11] may be used to supplement the proposal suggested here. However, it is evident that supporting evidence for or against a kinetic mechanism may be provided from initial rate studies with substrate inhibitors.

One obvious advantage of studying reaction mechanisms kinetically with substrate inhibitors is that definitive information on the nature of enzyme and substrate interactions can be obtained in most cases from experiments in a single direction only. This may be an important consideration when dealing with irreversible systems or with systems which form unstable products.

It is noteworthy that certain three substrate systems which give rise to two products[1,2] were not considered in the present report. Rate equations for these systems may be obtained by analysis outlined here or if studied from the reaction side involving two substrates, from an earlier publication[4].

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