CRITERIA FOR DISTINGUISHING BETWEEN THE RAPID EQUILIBRIUM ORDERED AND RANDOM BI BI KINETIC MECHANISMS*

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Summary

Three criteria are presented that permit a distinction to be made between the conventional rapid equilibrium Random Bi Bi mechanism and a unique Ordered Bi Bi mechanism in which the rapid equilibrium assumption is made [C. Frieden (1976) Biochem. Biophys. Res. Commun., 68, 914-917]. A choice between these mechanisms cannot be made using kinetic procedures now in vogue for differentiating between Ordered and Random Bi Bi mechanisms. However, transition state analogs, pulse-chase experiments, and pre-equilibrium isotope exchange procedures can be employed to make this distinction.

In 1953, Alberty (1) indicated how a choice could be made between Sequential[1] and Ping-Pong kinetic mechanisms from initial rate experiments. Within the Sequential class, two mechanisms, the rapid equilibrium Random Bi Bi and the steady-state Ordered Bi Bi, have received a good deal of attention from kineticists who have attempted to devise procedures in order to differentiate between them. These methods have been detailed elsewhere and will not be considered here (3). Suffice it to say that investigators have been reasonably confident, after conclusively eliminating the Ordered Bi Bi mechanism, that the Random Bi Bi case is the only reasonable alternative possible within the Sequential class. Frieden (4) has recently

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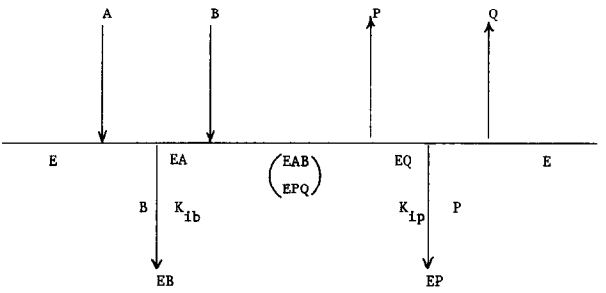
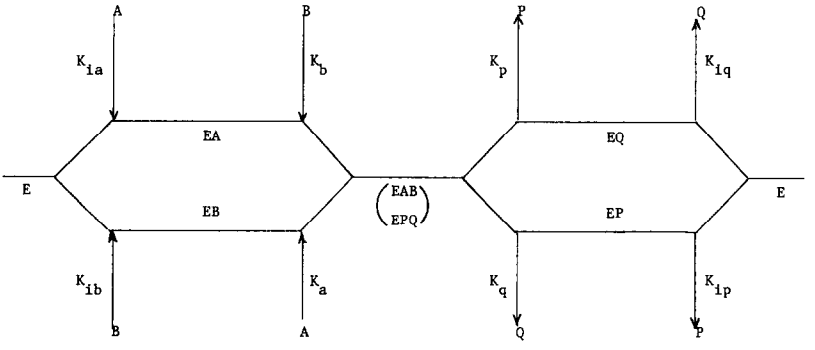
1 The nomenclature is that of Cleland (2).

proposed a modification of the Ordered Bi Bi mechanism, however, that provides initial-rate data that are indistinguishable from the rapid equilibrium Random Bi Bi mechanism. Frieden's proposal is outlined in Scheme I. In this pathway, EB and EP represent abortive complexes, and all steps are assumed to equilibrate rapidly relative to the interconversion of the productive ternary complexes.



Scheme I



Scheme II

Frieden (4) correctly points out that this model provides the same product inhibition patterns, alternative substrate inhibition patterns, substrate analog inhibition patterns, isotope exchange at equilibrium patterns, and Haldane relationship as the rapid equilibrium Random Bi Bi mechanism.

It is possible, however, to choose between this ordered mechanism and the random case illustrated in Scheme II by using one or more of at least three different kinetic criteria. The purpose of this report is to present those methods that permit this discrimination to be made.

One method that may be used to make a choice between these two mechanisms involves the use of multisubstrate analogs (transition state analogs). We have shown, for example, that, in the adenylate kinase reaction, the multisubstrate analog P^1, P^4-di(adenosine-5') tetraphosphate is a competitive inhibitor of both substrates (ATP and AMP) (5). It was pointed out at that time that such inhibition patterns are unique for the random mechanism because only the enzyme term, which is represented in the rate equation by the factor $K_{ia}K_b/V_1(A)(B)$, is inhibited by the analog; i.e., the analog binds both substrate sites simultaneously. The rate expression in this situation is shown in Equation 1, where K_i represents the dissociation constant for enzyme-inhibitor complex. In the bireactant steady-state Ordered mechanism the enzyme

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{(A)(B)}\left(1 + \frac{I}{K_i}\right)\right] \qquad (1)$$

term is made up of two factors in the rate equation, $K_{ia}K_b/V_1(A)(B)$ and $K_a/V_1(A)$. Thus, although the analog may be competitive with respect to substrate A, it will be noncompetitive relative to substrate B. In the case of Frieden's mechanism (4) depicted in Scheme I, the transition state analog should bind at both the active site and the subsite for B, i.e., the EB site. This will result in modification of a number of terms in the rate equation. The dead-end complexes to be expected are : EI_1, EI_2, EI_1I_2, and EAI_2, where I_1 and I_2 represent binding of the transition state analog at the active and subsites, respectively. The resulting rate expression is described by Equation 2 in which K_i, K_{ii}, K_{iii}, and K_{iv} represent dissociation constants for EI_1, EI_2, EI_1I_2, and EAI_2 complexes, respectively.

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{K_{ia}K_b}{K_{ib}(A)} + \frac{K_b}{B}\left(1 + \frac{I}{K_{iv}}\right) + \frac{K_{ia}K_b}{(A)(B)}\left(1 + \frac{I}{K_i} + \frac{I}{K_{ii}} + \frac{I^2}{K_iK_{iii}}\right)\right] \qquad (2)$$

Equation 2 indicates that the multisubstrate analog acts like a competitive inhibitor relative to substrate B and as a noncompetitive inhibitor with respect to substrate A. In addition, replots of the slopes from these primary plots versus inhibitor concentration will yield a concave-up parabola. This analysis indicates that a differentiation can be made between the mechanisms outlined in Schemes I and II.

Another procedure that may be employed to make a choice between the random and ordered mechanisms under discussion is of the pulse-chase type (6). In these experiments the enzyme is incubated with labeled substrate so as to form an enzyme-substrate complex. At time zero a mixture of unlabeled substrates (A + B) is added and the specific activity of the labeled product measured as a function of time. Using this method, it will be necessary to carry out two experiments, one involving labeled substrate A and the other involving labeled substrate B.

In the case of the Ordered Bi Bi mechanism (Scheme I), the specific activity of the product formed from labeled A will rise during the first turnover of the enzyme and then fall as unlabeled substrate A enters the product pool. On the other hand, when labeled substrate B is used, the specific activity of labeled product will remain constant with time. The rationale for this prediction is that the labeled substrate B associated with the enzyme will be at the subsite and not at the active site. Under these conditions the specific activity of the labeled product will remain constant during the first and subsequent turnovers of the enzyme. It is of interest that this same criterion may be used to determine the binding order of substrates in the steady-state Ordered Bi Bi mechanism.

In the case of the Random Bi Bi mechanism (Scheme II), the labeled substrate will always be at the active site of the enzyme prior to time zero,

and the specific activity pattern will first rise and then fall for both substrates. The uniqueness of these specific activity patterns with time permits a choice to be made between ordered and random kinetic mechanisms.[2]

The third method that may be used to differentiate between the pathways shown in Schemes I and II involves a procedure used by Kosow and Rose (7) to investigate the kinetic mechanism of yeast hexokinase. These workers added both substrates (ATP and glucose) to hexokinase and kept the concentration of one product essentially zero by using a coupling enzyme system (either NADP and glucose-6-P dehydrogenase or phosphoenolpyruvate and pyruvate kinase). They then added a labeled product (the one for which the coupling enzyme had not been added) and determined its rate of incorporation into substrate.

For the Ordered Bi Bi mechanism (Scheme I), if product Q is maintained at zero with coupling enzyme and labeled product P is added along with substrates (A + B), the label will appear in substrate B as a steady-state concentration of the binary complex EQ will be maintained. On the other hand, if labeled product Q is added with substrates and the level of product P kept at zero with the coupling system, the label from product Q will not appear in substrate A. When considering the Random Bi Bi pathway (Scheme II), the binary complexes EP and EQ will always be present when one of the products is removed with coupling enzyme. Thus either labeled product will be converted to substrate in the absence of the other product.

Although it may be difficult to use one or more of the methods proposed in this report to make a choice between the mechanisms of Schemes I and II, it is unlikely that technical problems will preclude the use of all three procedures. In light of Frieden's important contribution (4),

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[2]This treatment assumes that k_{off} for the labeled enzyme-substrate complexes in the rapid equilibrium mechanisms is not much greater than k_{cat}.

it seems reasonable to use some method to distinguish these possible mechanisms, if the rapid equilibrium Random Bi Bi pathway is inferred from initial rate studies of the type ordinarily carried out in the past.

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