ON THE RAPID EQUILIBRIUM ASSUMPTION AND THE PROBLEM OF DISTINGUISHING CERTAIN ORDERED AND RANDOM ENZYME KINETIC MECHANISMS*

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Received June 9,1977

Summary

Frieden [Biochem. Biophys. Res. Commun. (1976) 68, 914-917] recently identified limitations on using initial velocity data to distinguish certain ordered and random mechanisms. He found that the rapid equilibrium ordered mechanism with EB and EP abortive complexes cannot be distinguished from the random addition case, except by equilibrium exchange measurements. Re-examination of the rapid equilibrium assumption clearly demonstrates, however, that isotope exchange methods are also ineffective. Nonetheless, it is demonstrated that the rapid equilibrium assumption does not hold when the k_{cat} exceeds 30-50 sec^{-1}. Thus combined use of initial rates and isotope exchange approaches offer reliable tests for rigorously defining the mechanism of many, and possibly most, enzymes.

Determination of the substrate binding order in multisubstrate enzyme systems has been a topic of long-standing interest. Such information can often provide valuable evidence for the occurrence of ordered conformational changes evoked by interactions with substrates during catalysis. For this reason, considerable sustained effort has provided a theoretical framework for determining the kinetic mechanism by a number of approaches (1-3). Frieden (4), however, recently presented a cogent analysis suggesting that certain ordered and random mechanisms cannot be separated by initial rate methods. In particular, he cited the case of a rapid equilibrium ordered addition in which the second substrate to bind and first product to desorb during catalysis, B and P, can bind to uncomplexed enzyme to form EB and EP abortive complexes. Formation of such abortive binary complexes might be expected in the so-called ligand exclusion models (5) where A binds in a cleft and B binds over A to form EAB. He found that such a mechanism is indistinguishable from random addition cases by any of the initial rate methods, and concluded that equilibrium isotope exchange (3) is the only reliable means for choosing between these kinetic pathways.

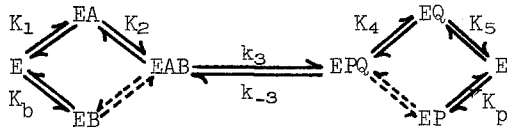
*Supported in part by Research Grant GM-21091 from the National Institutes of Health and by an appropriation from the Committee on Research, University of California, Santa Barbara, Ca.

ISSN 0006-291X

We show here that the isotope exchange studies would be equally ineffective if the ordered system noted above were truly rapid equilibrium. Nonetheless, a detailed examination of the rapid equilibrium assumption reveals that the mechanisms of many (and perhaps most) enzymes can be analyzed unambiguously by both initial rate and isotope exchange methods. A general approach for identifying the exceptions can be made by determining the magnitude of the catalytic constant (k_{cat}). For the cases where k_{cat} is less than 10 sec^{-1}, the rapid equilibrium ordered mechanism cited above cannot be rigorously excluded except under very special situations (6).

Theory

Consider the following mechanism in which A is the leading substrate in the sequential formation of the productive ternary complex:



K_1, K_2, K_4, K_5, K_b, and K_p are dissociation constants, and the rate constant of the slow step is either k_3 or k_{-3}, depending upon the reaction direction considered. The dotted arrows represent the additional pathways which do not occur in this mechanism but are common to the random addition pathway. Frieden (4) has shown that the rate expressions in the absence (Eqn.1) and presence (Eqn.2) of EB abortive formation are:

$$V_o = \frac{V_{mf}}{\left\{1 + \frac{K_2}{B} + \frac{K_1K_2}{AB}\right\}} \quad \text{(Eqn.1)} \qquad V_o = \frac{V_{mf}}{\left\{1 + \frac{K_2}{B} + \frac{K_1K_2}{K_bA} + \frac{K_1K_2}{AB}\right\}} \quad \text{(Eqn.2)}$$

One cannot uniquely distinguish Eqn.2 from the random pathway by initial rates, Haldane relationships, Dalziel φ relationships or the battery of inhibition techniques [including product inhibition, if EP forms] (4).

When the rapid equilibrium assumption is also applied in the derivation of isotope exchange rate equations, we find that this method also is incapable of providing a rigorous distinction. Boyer (3) correctly showed that steady state ordered mechanisms can be distinguished from random pathways by observing the effect on the A⇌Q exchange resulting from increases in the B and P concentrations in constant ratio. The following, however, is the rate law obtained for the A⇌Q exchange under the rapid equilibrium assumption:

$$R = k_3E_o \Big/ \left\{\frac{1}{(A)(B)}\left[1 + \frac{P}{K_p} + \frac{Q}{K_5}\right] + \frac{1}{K_B(A)} + \frac{1}{K_1(B)} + \frac{1}{K_1K_2} + \frac{K_{eq}}{K_4K_5}\right\} K_1K_2 \quad \text{(Eqn.3)}$$

Setting Q = αA and P = βB (such that $\alpha\beta = K_{eq}$), we get

$$R = k_3E_o \Big/ \left\{ \frac{1}{(A)(B)} \left[1 + \frac{\beta(B)}{K_p} + \frac{\alpha A}{K_5} \right] + \frac{1}{K_B(A)} + \frac{1}{K_1(B)} + \frac{1}{K_1K_2} + \frac{K_{eq}}{K_4K_5} \right\} K_1K_2 \qquad \text{(Eqn.4)}$$

Taking the limit as B goes to infinity, we find that

$$\lim_{B \to \infty} R = k_3E_o \Big/ \left\{ \frac{1}{(A)} \left[\frac{\beta}{K_p} + \frac{1}{K_B} \right] + \frac{1}{K_1K_2} + \frac{K_{eq}}{K_4K_5} \right\} K_1K_2 \qquad \text{(Eqn.5)}$$

This limit shows that as the level of B (and therefore P) is raised enormously high, the exchange rate reaches a maximum and will not decrease. One may anticipate this since A* is in rapid equilibrium with the EA and EAB forms and the gross exchange rate only depends upon k_3(EAB).

Conclusions based upon Eqns.2 and 3 can in fact be somewhat misleading because these rapid equilibrium cases are obtained by eliminating terms from the complete rate expressions. To circumvent this we have numerically evaluated the complete equation for the ordered mechanism with EB and EP abortives. The basic idea stems from the fact that the rapid equilibrium condition is valid only for certain values of the rate constants. For this reason, we began with the more general expressions for the rates at steady state (Eqn.6) and equilibrium (Eqn.7):

$$\frac{E_o}{v} = \frac{(E) + (EA) + (EAB) + (EPQ) + (EQ) + (EB) + (EP)}{k_3(EAB)} \qquad \text{(Eqn.6)}$$

$$\frac{R}{E_o} = \frac{k_1k_2k_3(A)(B)(E) \left\{ (k_{-1} + k_2B) - \frac{k_2k_{-2}B}{k_{-2}+k_3} \right\}^{-1}}{(E)+(EA)+(EAB)+(EPQ)+(EQ)+(EB)+(EP)} \qquad \text{(Eqn.7)}$$

The determinants for the various enzyme species were obtained using the ENZ EQ program of Fromm (1). These expressions can be examined by using values for rate constants to give rapid equilibration of all enzyme species except ternary complex interconversion. Bimolecular rate constants were set at $10^{7.5}$ M^{-1} sec^{-1}, which are reasonable values for enzyme-substrate reactions (7). With the exception of ternary complex interconversion, the unimolecular rate constants were set at $10^{3.5}$ sec^{-1}, and thus all dissociation constants are 10^{-4} M. The ternary complex interconversion was described by various values. Of course, there were so many combinations of rate constants which we considered, but these are quite representative.

It was noted that for k_3 values of around 20 sec^{-1} or less, initial rate plots in the absence of EB and EP complex formation were characteristic of equilibrium ordered mechanisms (i.e., there was a characteristic convergence

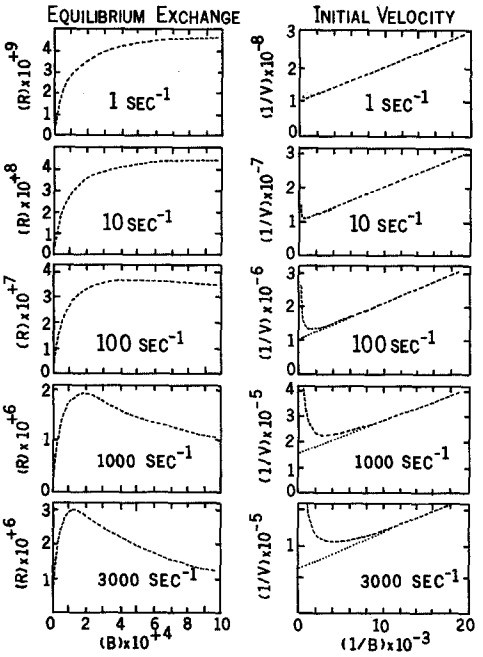


Figure 1 : Comparison of equilibrium exchange rates (R) and initial velocity (v) measurements for various values of the rate constants for ternary complex interconversion. Total enzyme was held at 20 nM; the bimolecular rate constants were $10^{7.5}$ M^{-1} sec^{-1} and the unimolecular constants were $10^{8.5}$ sec^{-1}. For the equilibrium rate calculations, A and Q were maintained at 0.1 mM (K_m levels), and the absolute levels B and P were raised in a constant ratio of unity as shown on the graph. For the initial velocity computations, A was maintained at 0.05 mM (1/2 K_m), and P and Q were zero.

of all lines at the 1/v-axis in plots of 1/v versus 1/B). However, by including EB and EP abortives, all plots give convergence to the left of the (1/v)-axis, and this shows that the general model behaves as predicted by Frieden's rapid equilibrium equation (4). The important finding of this study is shown in Fig.1, where the A ⇌ Q equilibrium exchange rate and the initial velocity plots are compared at various values of k_3 and k_{-3}. When k_3 is fairly small (i.e., 10 sec^{-1} or less), one must raise B and P to levels corresponding to 10-20 times K_b and K_p to observe any depression in the exchange. As k_3 gets greater than 30-50 sec^{-1} the steady state assumption becomes relevant, and the A ⇌ Q exchange is depressed by raising B and P. Significantly, the initial velocity plots show a strong substrate inhibition, characteristic of EB abortive formation, at or above such k_3 values.

The applicability of the rapid equilibrium assumption thus depends on the

TABLE I : Catalytic Constants of Some Multisubstrate Enzymes†

Enzyme	$k_{cat}(sec^{-1})$	Ref.	Enzyme	$k_{cat}(sec^{-1})$	Ref.
Yeast Hexokinase	230-750	a	Acetate Kinase	180-240	l,m
Carbamate Kinase	1170	b	Liver Alcohol Dehydrogenase	125	n
Muscle Phosphofructokinase	890	c	Yeast Alcohol Dehydrogenase	3850	o
Adenylate Kinase	780	d	Malate Dehydrogenase	335	p
Yeast Phosphoglycerate Kinase	900	e	α-Glycero-P Dehydrogenase	130	q
Muscle Pyruvate Kinase	300	f	Heart Lactate Dehydrogenase	260	r,s
Creatine Kinase	330	g	Liver Glutamate Dehydrogenase	30	t
Brain Glutamine Synthetase	186	h	Lactate Oxidase	104	u
Succinyl-CoA Synthetase	30	i	Prolyl Hydroxylase	103	v
Acetyl-CoA Synthetase	33	j	Glucose Oxidase	1150	w
Beef Heart F_1 ATPase	100	k	Superoxide Dismutase	1650	x

†Estimated from initial rate data.

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rate of the ternary complex step. By definition, the rate constant for this step is k_{cat} in either the rapid equilibrium random or the Frieden mechanism. Since initial rate or isotope exchange methods can easily distinguish these mechanisms for k_{cat} values above 30-50 sec^{-1} (see Fig.1) the dilemma pointed out by Frieden (4) is not at all general. Indeed, the k_{cat} values of a number of enzyme systems are clearly considerably above this range (Table I). Undoubtedly, there are a number of enzymes with lower catalytic constants, and these will not submit to a definitive distinction by these approaches. Nonetheless, knowledge of the k_{cat} value can be used in conjunction with the plots like those in Fig.1. If the k_{cat} is above 30-50 sec^{-1}, then the ordered mechanism with EB and EP abortives will be discernible by the characteristic substrate inhibition and depressed equilibrium exchange rates. Both phenomena will be measurable at experimentally accessible values of B and P.

Concluding Remarks

For reasons cited above, the dilemma encountered in distinguishing certain ordered and random addition mechanisms is not a general problem. Our analysis suggests that the combined use of isotope exchange and initial rates can provide a reliable general test of mechanism whenever k_{cat} exceeds 30-50 sec^{-1}. With initial rate studies alone, one may not distinguish the true nature of the substrate inhibition, but one learns more by combination with isotope exchange. For example, substrate inhibition will occur with both exchanges if dead end complexes are formed, but only one exchange will be depressed with the ordered pathway.

One might, of course, extend the generality of our analysis by considering the relative magnitudes of rate constants in a pair-wise fashion. For example, an important case might deal with k_{cat} compared to k_2, the rate constant for EAB $\rightarrow$ EA + B. There are many such comparisons, but the basic ideas presented here will still be valid. The key point is that the rapid equilibrium assumption must be valid for Frieden's dilemma (4) to be considered, and k_{cat} must be very small. For such cases, Fromm (6) has suggested three experimental criteria, but their general value in discerning the mechanism is not yet well documented. There are additional problems with some of these depending upon the topology of the active site. Nonetheless, it is noteworthy that there are no apparent thermodynamic procedures for answering the problem. In this respect, the dilemma posed by Frieden (4) is a limiting case which constitutes a kinetic problem, requiring some new and experimentally reliable kinetic protocols for its solution.

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