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KINETIC DISTINCTION BETWEEN RAPID-EQUILIBRIUM RANDOM AND ABORTIVE ORDERED ENZYMATIC MECHANISMS USING ALTERNATIVE SUBSTRATES OR KINETIC ISOTOPE EFFECTS

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SUMMARY: Alternative substrates, such as those isotopically-labeled, which differ in their rate constants of catalysis but not in their rate constants of binding, generate identical values of V/K_a in ordered kinetic mechanisms of bireactant enzymes. This is shown to be true even for the rapid-equilibrium ordered mechanism in which an abortive complex between free enzyme and the second substrate is formed. In contrast, rapid-equilibrium random mechanisms have non-identical values for V/K_a. Consequently, the effect of alternative substrates or isotope effects on V/K_a provides a means to distinguish between these nearly identical kinetic mechanisms.

Introduction: Kinetic characterization of enzyme mechanisms frequently involves the use of initial velocity experiments to distinguish between ping-pong and sequential mechanisms. Within the sequential class of bisubstrate enzyme-catalyzed reactions are those in which the substrates must bind in compulsory order prior to catalysis and those in which the substrates may bind to the enzyme randomly. Alberty (1) demonstrated that the derivation of an ordered substrate addition using the steady-state assumption and random substrate addition using the rapid equilibrium assumption resulted in the same expression for initial velocity dependence as shown in Eq. 1:

$$v = \frac{V[A][B]}{K_a[B] + K_b[A] + K_{ia}K_b + [A][B]} \qquad (1)$$

in which A and B are the substrates, K_a and K_b are Michaelis constants, K_{ia} is a dissociation constant, and V is the maximum velocity. Thus, ordered versus random mechanisms cannot be distinguished on the basis of initial velocity patterns alone. Instead, alternative kinetic methods were developed for this purpose, including prod-

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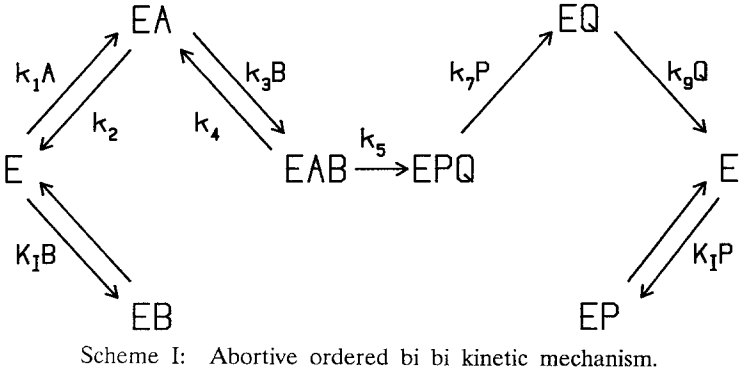
uct inhibition (2,3), use of alternative substrates as inhibitors or competitive inhibitors (4,5) and isotope exchange experiments (6). However, Friedan (7) noted that, under rapid-equilibrium conditions, an abortive ordered mechanism could not be distinguished from a random one by these means. The former mechanism, also termed an ordered bi bi - subsite mechanism (8), involves ordered binding of substrates leading to catalytic turnover but has an additional and abortive enzyme-second substrate complex (and analogously, an abortive enzyme-first product complex) as shown in scheme I. The mechanism is described by the initial velocity equation:

$$v = \frac{V\,[A][B]}{K_a[B](1 + [B]/K_I) + K_b[A] + K_{ia}K_b(1 + [B]/K_I) + [A][B]} \qquad (2)$$

in which K_I is the dissociation constant of the abortive complex.

The kinetic problem can be grasped intuitively by imagining reversible steps connecting EA to EAB, and EPQ to EP; under equilibrium conditions, the presence or absence of these steps is kinetically insignificant because alternative routes exist. Nevertheless, determining their presence or absence is a significant kinetic question, because the answer can have a bearing on possible chemical mechanisms and on designs of inhibitors. Fromm (9) addressed the question and presented three methods to detect the missing steps: use of transition-state analog inhibitors, pulse-chase labeling experiments, and non-equilibrium isotope exchange. Although useful in many cases, the procedures all entail limitations in experimental design and cannot be universally applied. We now present a fourth method of similar design to initial velocity kinetics, based upon the use of an alternative substrate or an isotope effect (which derives from a special kind of alternative substrate).

Theory: Radika and Northrop (10) determined that if the concentration of substrate A were varied at fixed and saturating concentrations of substrate B or its



Scheme I: Abortive ordered bi bi kinetic mechanism.

alternates B' or B", and similarly, B were varied against A, A', or A", that unique pairs of kinetic patterns of double reciprocal plots would be generated by ordered

and random kinetic mechanisms. The critical kinetic parameter is the apparent V/K_a, which is independent of B in ordered kinetic mechanisms, but dependent upon B in random kinetic mechanisms.

For the abortive ordered mechanism, Eq. 2 may be written in reciprocal form as shown in Eq. 3:

$$\frac{1}{v} = \left[\frac{K_a}{V} + \frac{K_a[B]}{VK_I} + \frac{K_{ia}K_b}{VK_I}\right]\left[\frac{1}{[A]} + \frac{K_b}{V[B]} + \frac{K_{ia}K_b}{V[A][B]}\right] + \frac{1}{V} \qquad (3)$$

The apparent V/K_a at high [B] is:

$$\left[\frac{V}{K_a}\right]_{app} = \frac{V}{K_a} + \frac{VK_I}{K_a[B]} + \frac{VK_I}{K_{ia}K_b} \qquad (4)$$

Under rapid-equilibrium conditions (i.e. $k_5 << k_4$) it becomes[2]:

$$\left[\frac{V}{K_a}\right]_{app} = \frac{VK_I}{K_{ia}K_b} = \frac{k_1k_3K_I}{k_2} \qquad (5)$$

Hence, because of the presence of K_I, V/K_a in the abortive ordered mechanism is dependent upon the identity of substrate B, as is also the case in random mechanisms, but with a difference: in the former, only a change in B which alters binding to the enzyme will express a change in V/K_a, whereas in the latter, changes in binding and changes in catalysis will be expressed.

Experimental Design: In order to invoke the rapid-equilibrium assumption, values of V/K_b should be significantly below the expected diffusion-controlled rate of combination of substrate with enzyme. The use of Eq. 5 to test whether an alternative substrate B changes the apparent V/K_a requires access to and knowledge of alternative substrates which differ in catalysis but not in binding. This question is easily addressed when dealing with a rapid-equilibrium mechanism because K_b is then a dissociation constant and V is a direct measure of catalysis. The clearest example of such an alternative is an isotopically-labeled substrate, because the presence of isotopes does not change non-covalent binding of substrates to enzymes (11). Using a fully deuterated substrate, it makes no difference which substrate carries the label; one simply proceeds within a non-competitive experimental design (12) and determines whether an isotope effect is expressed on V/K_a. Using a trace label, it is necessary that the isotope be carried on substrate A. In both, the

2. Only the rapid-equilibrium mechanism is of interest because under steady-state conditions, Eq. 3 predicts a highly diagnostic pattern of substrate inhibition by B which is competitive with A.

expression of an isotope effect on V/K_a is dependent upon the forward commitment to catalysis, which in an ordered mechanism is:

$$C_f = \frac{k_5}{k_4}\left[1 + \frac{k_3[B]}{k_2}\right] \qquad (6)$$

Northrop (13) noted that in an ordered but not a random mechanism, saturation with B will raise the forward commitment to catalysis and consequently abolish the expression of an isotope effect on V/K_a. In an abortive ordered mechanism, the formation of EB removes enzyme from catalytic turnover but does not alter the sequence of steps linking A to EPQ; hence, the commitment to catalysis of A remains unchanged as does the expression of the isotope effect. Therefore, in the abortive ordered mechanism, saturation with B will also abolish the expression of an isotope effect on V/K_a.

Applications: V/K_a values of Mg:ATP for 2"-aminoglycoside nucleotidyltransferase were determined using three alternative aminoglycoside antibiotics, and found to differ by less than two-fold while the respective V/K_b values of the three aminoglycosides differed by over 100-fold (14). That the kinetic mechanism is ordered was confirmed by substrate and dead-end inhibition, and by viscosity- and pH-dependent kinetics. That an abortive EB complex is formed was evidenced by tight-binding to aminoglycoside affinity columns in the absence of nucleotide and by the partial character of uncompetitive substrate inhibition, indicating the breakdown of an EQB complex to EB.

V/K_a values of acetyl-coenzyme A for 3N-aminoglycoside acetyltransferase were similarly determined with alternative aminoglycosides, and also found to differ by about two-fold (15). Unfortunately, the V/K_b values of the four aminoglycosides employed differed by only three-fold, so it could not be ascertained whether the changes originated from a common origin (signifying a random mechanism) or if the former derived from experimental error and the latter from catalysis (consistent with an ordered mechanism). However, a solvent isotope effect arises in catalysis from H/D exchange on the 3-amino group of aminoglycoside substrates, leaving an N-D bond which must be cleaved during acetylation. Using protonated and deuterated sisomicin as alternative substrates, a value of 5.1 ± 0.3 was obtained for $^{D}(V/K_b)$, extrapolated to infinite acetyl-CoA, whereas a value of 0.96 ± 0.10 was obtained for $^{D}(V/K_a)$, extrapolated to infinite sisomicin, providing unequivocal proof of an ordered addition of nucleotide first and aminoglycoside second. That an abortive EB complex is possible with this enzyme was similarly evidenced by tight-binding to aminoglycoside affinity columns in the absence of nucleotide.

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