Kinetics of enzymes with iso-mechanisms: analysis of product inhibition

Karen L. REBHOLZ and Dexter B. NORTHROP*

Division of Pharmaceutical Biochemistry, School of Pharmacy, University of Wisconsin, Madison, WI 53706, U.S.A.

Isomerizations of free enzyme can be detected in kinetic patterns of product inhibition when the isomerization is partially rate-limiting. The kinetic pattern is non-competitive, owing to binding of substrate and product to different forms of free enzyme. This adds an additional term to the rate equation, sometimes represented as K_{SP} . Several kineticists have noted that, as the rate of isomerization becomes high in relation to catalytic turnover, the intercept effect will become small, K_{SP} will approach infinity, and the pattern will look competitive. Britton [(1973) *Biochem. J.* 133, 255–261] asserted that K_{SP} will also approach infinity when the rate of isomerization becomes low. This second assertion is incorrect and can be traced to the particular model and graphical

representation used to examine K_{SP} as a function of relative rate constants. The function portrayed as a parabola with two roots for K_{SP} is, instead, a straight line with one root. The algebraic condition justifying the second root obtains in the limit of zero in the rate of reaction and thus is not experimentally relevant, and the appearance of competitive inhibition, based on K_{SP} alone, is not valid. Using a more general model, new equations are derived and presented which provide direct calculations of the apparent rate constants for free enzyme isomerizations from product-inhibition data when the equilibrium of the isomerization is near 1, and useful limits for the rate constants when greater than or less than 1.

INTRODUCTION

The possibility of enzymes finishing the chemical segment of catalysis in a form different from that in which they started has a long theoretical history (Medwedew, 1937; King, 1956; Botts, 1958; Cleland, 1963; Britton, 1973; Fromm, 1975). Called 'iso-mechanisms' in reference to a necessary isomerization of free enzyme, this kinetic problem was first described in terms of the kinetics of product inhibition. In the absence of an iso-mechanism, the last product is a competitive inhibitor of the first substrate, because both reactants compete for the same form of enzyme. In contrast, when isomerizations occur and the first substrate and last product bind to different forms of enzyme, product-inhibition patterns should be non-competitive with intercept effects that are related in some way to the rates of isomerization.

Experimentally, however, distinguishing between competitive and non-competitive product inhibition has been elusive. The standard experimental design is to construct double-reciprocal plots of initial velocities as a function of substrate concentrations at different concentrations of product inhibitor. Competitive product inhibition generates a pattern of lines intersecting on the vertical axis, while non-competitive product inhibition intersects to the left of the vertical axis. Hence the objective within the kinetics of iso-mechanisms is to determine the presence or absence of an intercept effect. The effect is accentuated at high concentrations of substrate and product, and increasing both to extreme levels is often necessary. For some enzymic reactions, however, accentuated conditions may not be attainable because of solubility or absorbency limits. Furthermore, Darvey (1972) realized that this design could not be used at all with reversible Uni Uni reactions because individual lines are curved and thus cannot be extrapolated to the vertical axis.

Britton (1973) understood these limitations and formulated an ingenious experimental design which he termed 'induced transport' to detect isomerizations of free enzyme. It involves a perturbation of isotopic equilibrium of labelled substrate and

product, and, from the maximum perturbation, Britton (1973) extracted a kinetic function termed α , which he attempted to relate to the steady-state rate equations for non-competitive product inhibition. However, Britton (1973) was frustrated by the lack of a definite relationship between his kinetic constants and the relative rates of isomerizations. We now show that his frustration derives from two errors in his analysis, and present an expanded derivation which provides the definition that Britton (1973) sought.

THEORY

Steady-state rate equations of Britton (1973)

Britton (1973) formulated the kinetic problem with the following reaction mechanism:

Scheme 1

The steady-state velocity in the presence of inhibitory product was presented in the following form:

$$v = \left[\frac{\frac{V_s}{K_s} (1 + \alpha K_{eq} [P]) [S] [E]}{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[S][P]}{K_{SP}}} \right] - \left[\frac{\frac{V_p}{K_p} (1 + \alpha [S]) [P] [E]}{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[S][P]}{K_{SP}}} \right] \quad (1)$$

* To whom correspondence should be addressed.

Britton derived the following relationship from a series of expressions of individual rate constants in terms of kinetic constants:

$$K_{SP} = \frac{V_S K_P}{\alpha (V_S + V_P - \alpha K_S V_P)} \quad (2)$$

where:

$$\alpha = \frac{k_{+1} k_{+2}}{k_{-3} (k_{-1} + k_{+2})} \quad (3)$$

$$K_{SP} = \frac{(k_{+3} + k_{-3})(k_{-3} + k_{+2})}{k_{+1} k_{-2}} \quad (4)$$

Britton (1973) noted that, when the isomerization is very rapid and both k_{+3} and k_{-3} approach infinity, α will approach zero, K_{SP} will approach infinity and the $[S][P]/K_{SP}$ term in the denominator of the steady-state equation will disappear, adding that this conclusion had been reached by others (Cleland, 1963; Ray and Roscelli, 1964; Taraszka and Alberty, 1964). He went on to say that "... K_{SP} will also approach infinity and the $[S][P]/K_{SP}$ term will disappear from the denominator of eqn. (1) when α approaches the equality:

$$\alpha = (V_S + V_P)/(K_S V_P) \quad (5)$$

Britton also noted that "... α can only approach the equality ... as a limiting case. This corresponds to the condition that k_{+3} and k_{-3} are finite and that k_{-1} and k_{+2} approach infinity. The $[S][P]/K_{SP}$ term therefore also disappears from the denominator of the steady-state rate equation (eqn. 1) when the isomerization is the major rate-limiting step in the reaction." Because "The conclusion that the absence of an $[S][P]/K_{SP}$ term in the denominator does not necessarily mean that any isomerization of the enzyme is rapid may seem surprising." Britton illustrated the relationship between K_{SP} and the rate constants for isomerization with a numerical simulation with $k_{+1} = k_{-2}$, and $k_{+3} = k_{-3} = mk_{-1} = mk_{+2}$ which produced a parabola. Britton noted that "Not only does K_{SP} approach infinity both when $m \rightarrow 0$ and $m \rightarrow \infty$, but the parabolic shape of the curve means that in general two values of m may be found to satisfy a given of K_{SP} ."

Attempts to reconstruct Britton's numerical example proved to be less than straightforward. Figure 1 shows a plot of values of K_{SP} versus values of m when k_{+3} and k_{-3} are varied by factors of 2 from 0.015625 (i.e. 1/64, see below) to 64 while the other rate constants are fixed at 1. A linear relationship is generated, not a parabolic one. Returning to Britton's (1973) Figure 1, it now becomes apparent that the scale on the ordinate is exponential; each successive unit division on Britton's graph represents a power of 2 over the previous value. The numerical data of Figure 1 were replotted on Britton's (1973) axis, as shown in Figure 2. Only half of Britton's Figure 1 is reconstructed, but it clearly shows that the 'parabolic' function was an artifact of the exponential scaling.

The peculiarity of the scaling suggests an origin for the second half of Britton's (1973) curve; either the numerator or denominator rate constants of m can be changed to generate the ordinate values. Figures 1 and 2 were obtained by varying the numerator; so as an alternative, a second simulation was generated by varying the denominator, as shown in Figure 3. The curve corresponds to the missing half of the parabola and shows that the second limit is also an artifact of the scaling. Britton's (1973) Figure 1 was supposed to represent a single phenomenon, a relative change in the rate constants for isomerization, but by varying k_{+2} and k_{-1} while holding k_{+1} and k_{-2} constant, the dissociation constants for S and P were changed and the latter

Figure 1 An attempted reconstruction of Britton's (1973) Figure 1 varying only k_{+3} and k_{-3} as a means to change m , plotted on a normal scale

Figure 2 A replot of Figure 1 using Britton's scaling for m

Figure 3 An attempted reconstruction of Britton's (1973) Figure 1, varying k_{+2} and k_{-1} as a means to change m using his scaling for m

directly changes K_{SP} . The converse is certainly not true in a more general mechanism; that is, changes in dissociation constants need not necessarily cause a change in the kinetic significance of an isomerization. It is unfortunate that Britton (1973) restricted

himself to the mechanism in Scheme 1, which omits the chemical conversion of ES into FP. If this step had been included and assigned rate constants, then the second simulation could have been undertaken without altering dissociation constants; Britton might then have found the unique solution for which he was looking.

Eqn. (5) was offered as descriptive of the second, 'artifactual', limit for large values of K_{sp} . The numerical simulations revealed, in addition to the above, that the equivalence shown in eqn. (5) obtains in the first simulation as the velocity of the reaction goes to zero (a result of an approach to dividing a zero by another zero). The case described by Britton, i.e. " k_{-1} and k_{+2} approach infinity" is a limit from the second simulation, in which dissociation constants are changing in addition to the kinetic significance of the isomerization. Hence, eqn. (5) has no relevance to the kinetics of iso-mechanisms.

Multi-step reversible Iso Uni Uni reactions

In order to obtain a general rate equation and avoid errors, both the chemical segment and isomerization segment should contain more than a single reactive step. The derivations which follow consider a minimal iso-mechanism with two steps in the chemical segment and two steps in the isomerization segment:

Scheme 2

The derivation below assumes that the isomerizations occur after release of the product. It is possible that isomerizations could occur before product release, but then the reaction would not be described as an iso-mechanism. The nomenclature for kinetic constants and enzyme forms is derived from the standard suggested by Cleland (1963); the form of enzyme that binds substrate is labelled E, the form of enzyme that is initially present after product release is labelled F, and additional intermediate forms labelled consecutively with letters starting with G (excluding I).

Eqn. (6) is a translation of eqn. (1) describing the steady-state velocity for an Iso Uni Uni mechanism (Britton, 1966, 1973):

$$v = \left[\frac{V_t \left(1 + \frac{\alpha_{iso}[P]}{K_{eq.}} \right) [A]}{1 + \frac{[A]}{K_a} + \frac{[P]}{K_p} + \frac{[A][P]}{K_{ip}K_a}} \right] - \left[\frac{\frac{V_r}{K_p} (1 + \alpha_{iso}[A])[P]}{1 + \frac{[A]}{K_a} + \frac{[P]}{K_p} + \frac{[A][P]}{K_{ip}K_a}} \right] \quad (6)$$

Catalytic turnovers are represented by a maximal velocity, e.g. V_t , in part for algebraic simplicity, but are equivalent to $V/[E_t]$ or $k_{cat.}$ in this discussion. (References to the concentration of enzyme were left out to emphasize that the measures of how rate-limiting an isomerization segment is, represented by eqns. 31 and 33 below, are independent of the amount of enzyme present in an experiment.) Note that Britton's K_{sp} is replaced by Cleland's $K_{ip}K_a$, Britton's α is replaced by α_{iso} , one of two roots of the quadratic equation (see below), and $K_{eq.}$ is now $1/K_{eq.}$. Eqn. (6) may be simplified to the standard form suggested by Cleland (1963):

$$v = \frac{V_t V_r \left([A] - \frac{[P]}{K_{eq.}} \right)}{K_a V_r + V_r [A] + \frac{V_t [P]}{K_{eq.}} + \frac{V_r [A][P]}{K_{ip}}} \quad (7)$$

Notice that, in eqn. (7), the α_{iso} term has disappeared; it therefore must represent a combination of steady-state kinetic constants (see eqn. 25 below).

To understand the relationship between the α of induced transport and steady-state kinetic constants, it is necessary to examine the limits and components of the latter. The maximum velocity in the forward direction, V_t , is composed of apparent rate constants which represent the chemical and isomerization segments, k_{tchem} and k_{tiso} respectively:

$$V_t = \frac{1}{\frac{1}{k_{tchem}} + \frac{1}{k_{tiso}}} \quad (8)$$

The apparent rate constants may be replaced by their component net rate constants [Cleland (1975); indicated by primes]:

$$V_t = \frac{1}{\frac{1}{k'_3} + \frac{1}{k'_5} + \frac{1}{k'_7} + \frac{1}{k'_9}} \quad (9)$$

or by the individual rate constants of Scheme 2:

$$V_t = \frac{k_3 k_5 k_7 k_9}{k_3 k_5 (k_7 + k_8 + k_9) + k_7 k_9 (k_3 + k_4 + k_5)} \quad (10)$$

Similarly, the maximum velocity in the reverse direction is labelled V_r and is comprised of k_{rchem} and k_{riso} :

$$\begin{aligned} V_r &= \frac{1}{\frac{1}{k_{rchem}} + \frac{1}{k_{riso}}} = \frac{1}{\frac{1}{k'_2} + \frac{1}{k'_4} + \frac{1}{k'_6} + \frac{1}{k'_{10}}} \\ &= \frac{1}{\frac{k_2 + k_3 + k_4}{k_2 k_4} + \frac{k_8 + k_9 + k_{10}}{k_8 k_{10}}} \\ &= \frac{k_2 k_4 k_8 k_{10}}{k_2 k_4 (k_8 + k_9 + k_{10}) + k_8 k_{10} (k_2 + k_3 + k_4)} \end{aligned} \quad (11)$$

The effect of the isomerization segment on the definitions of V_t and V_r is a kinetic function, as shown by eqns. (8)–(11). In contrast, the effect of the isomerization segment on the definitions of V_t/K_a and V_r/K_p is a thermodynamic function, because V/K expresses catalysis at extremely low concentrations of substrate and isomerizations of free enzyme are at equilibrium. The fraction of free enzyme present as form E, f_E , which can react with substrate, and form F, f_F , which can react with product, are shown in eqns. (12) and (13):

$$f_E = \frac{k_7 k_9}{k_7 k_9 + k_7 k_{10} + k_8 k_{10}} \quad (12)$$

$$f_F = \frac{k_8 k_{10}}{k_7 k_9 + k_7 k_{10} + k_8 k_{10}} \quad (13)$$

V_t/K_a is defined as the product of k'_1 and f_E :

$$\frac{V_t}{K_a} = k'_1 f_E = \frac{k_1 k_3 k_5 k_7 k_9}{(k_2 k_4 + k_2 k_5 + k_3 k_5) (k_7 k_9 + k_7 k_{10} + k_8 k_{10})} \quad (14)$$

Similarly, V_r/K_p is defined as the product of k'_6 and f_F :

$$\frac{V_r}{K_p} = k'_6 f_F = \frac{k_2 k_4 k_6 k_8 k_{10}}{(k_2 k_4 + k_2 k_5 + k_3 k_5) (k_7 k_9 + k_7 k_{10} + k_8 k_{10})} \quad (15)$$

A distinction must be made between the equilibrium constant for the entire reaction, $K_{eq.}$, and the equilibrium constant for the isomerization segment, $K_{eq.(E/F)}$. $K_{eq.}$ contains the rate constants from both the chemical and isomerization segments of the reaction as expressed in the Haldane relationship:

$$K_{eq.} = \frac{V_t K_p}{K_a V_r} = \frac{k_1 k_3 k_5 k_7 k_9}{k_2 k_4 k_6 k_8 k_{10}} \quad (16)$$

whereas $K_{eq.(E/F)}$ contains only the rate constants in the isomerization segment:

$$K_{eq.(E/F)} = \frac{f_E}{f_F} = \frac{k_7 k_9}{k_8 k_{10}} \quad (17)$$

K_{lip} , the non-competitive inhibition term for the product, is defined as follows:

$$K_{lip} = \frac{k_3 k_5 (k_7 + k_8 + k_9) + k_7 k_9 (k_3 + k_4 + k_5)}{k_6 (k_3 + k_4) (k_8 + k_9)} \quad (18)$$

A similar non-competitive inhibition term, K_{lia} , is found in the kinetics of the reverse reaction and is related to the first by a second Haldane relationship, unique to iso-mechanisms:

$$K_{eq.} = \frac{V_t K_{lip}}{V_r K_{lia}} \quad (19)$$

Britton's α is related to K_{lip} as shown in eqn. (20) (Britton, 1973):

$$K_{lip} = \frac{V_t K_p}{\alpha K_a (V_t + V_r - \alpha K_a V_r)} \quad (20)$$

Eqn. (20) is quadratic in α as shown upon rearrangement:

$$\alpha^2 \left(\frac{K_a}{V_t} \right) - \alpha \left(\frac{1}{V_t} + \frac{1}{V_r} \right) + \frac{K_{eq.}}{K_{lip} V_t} = 0 \quad (21)$$

When eqn. (21) is solved for α using V_t , V_r , K_a , $K_{eq.}$, and K_{lip} , two roots are obtained, and both roots can be used to study the kinetic importance of the isomerization segment of an enzymic reaction.

The first root was recognized by Britton (1973) and is here termed α_{iso} because of its relation to the isomerization segment as shown in eqn. (22):

$$\begin{aligned} \alpha_{iso} &= \frac{V_t}{K_a} \left(\frac{1}{k_{fiso}} + \frac{1}{K_{riso}} \right) \\ &= \frac{k'_1}{k'_{10}} = \frac{k_1 k_3 k_5 (k_8 + k_9)}{k_8 k_{10} (k_2 k_4 + k_2 k_5 + k_3 k_5)} \\ &= \frac{k_2 k_4 k_6 K_{eq.} (k_8 + k_9)}{k_7 k_9 (k_2 k_4 + k_2 k_5 + k_3 k_5)} = \frac{k'_6 K_{eq.}}{k'_7} \end{aligned} \quad (22)$$

α_{iso} is derived from the net rate constant for the first step in the reaction divided by the net rate constant for the last reverse step in the isomerization segment. As the first net rate constant of isomerization, k'_7 , increases relative to the first net rate constant for the back reaction, k'_6 , thereby making the mechanisms less iso, α_{iso} becomes smaller. Conversely, as the rate of conversion of F into E decreases relative to the back reaction, making the

mechanism more iso, α_{iso} increases. As alternatives to α_{iso} , Albery and Knowles (1987a) use c_p and Cleland (1990) uses x . The relation between α_{iso} , c_p , and x is:

$$\alpha_{iso} = \frac{1 + K_{eq.}}{c_p} = \frac{x(1 + K_{eq.})}{A_0 + P_0} \quad (23)$$

The second root of α from eqn. (21) is termed α_{chem} because of its relation to the chemical segment:

$$\begin{aligned} \alpha_{chem} &= \frac{V_t}{K_a} \left(\frac{1}{k_{fchem}} + \frac{1}{k_{rchem}} \right) \\ &= \frac{k_1 k_7 k_9 [k_2 k_4 (k_3 + k_4 + k_5) + k_3 k_5 (k_2 + k_3 + k_4)]}{k_2 k_4 (k_2 k_4 + k_2 k_5 + k_3 k_5) (k_7 k_9 + k_7 k_{10} + k_8 k_{10})} \end{aligned} \quad (24)$$

α_{iso} and α_{chem} are related:

$$\alpha_{iso} + \alpha_{chem} = \frac{1}{K_a} + \frac{K_{eq.}}{K_p} \quad (25)$$

Comparing eqns. (22), (24) and (25), it becomes obvious that if the chemical and isomerization segments are equally rate-limiting, then α_{iso} will equal α_{chem} . In most cases iso-mechanisms are barely detectable, the isomerization is only partially rate-limiting, and α_{iso} is much larger than α_{chem} ; hence, the larger root of eqn. (21) is usually associated with the isomerization segment.

Determining the rate constants for isomerization

Rearranging eqn. (22) isolates the apparent rate constants for the isomerization segment in the form of reciprocals:

$$\frac{\alpha_{iso}}{V_t/K_a} = \frac{1}{k_{fiso}} + \frac{1}{k_{riso}} \quad (26)$$

Similarly, the kinetics for the chemical segment can be expressed as:

$$\frac{\alpha_{chem}}{V_t/K_a} = \frac{1}{k_{fchem}} + \frac{1}{k_{rchem}} \quad (27)$$

Britton (1973) derived a relationship similar to eqn. (26) for Scheme 1, but erroneously described the left-hand portion in reciprocal form, i.e. as $V_s/\alpha K_s$, and did not attempt to extract individual rate constants from it. If the isomerization is represented by a single step, or if intermediates such as form G of Scheme 2 are present in minor amounts, then $K_{eq.(E/F)} = k_{fiso}/k_{riso}$. When $K_{eq.(E/F)} = 1$, then $k_{fiso} = k_{riso} = (V_t/K_a)/2\alpha_{iso}$. If $K_{eq.(E/F)}$ is not equal to 1, but is nevertheless known, it is possible to calculate the values of k_{fiso} and k_{riso} from the following relationships:

$$k_{fiso} = (K_{eq.(E/F)} + 1) \left(\frac{1}{1/k_{fiso} + 1/k_{riso}} \right) \quad (28)$$

$$k_{riso} = (1 + 1/K_{eq.(E/F)}) \left(\frac{1}{1/k_{fiso} + 1/k_{riso}} \right) \quad (29)$$

If $K_{eq.(E/F)}$ is much less than one, then eqn. (26) reduces to:

$$k_{fiso} = \frac{V_t/K_a}{\alpha_{iso}} \quad (30)$$

or if much greater than one, then:

$$k_{riso} = \frac{V_t/K_a}{\alpha_{iso}} \quad (31)$$

When $K_{\text{eq.}(E/F)}$ is not equal to $k_{\text{fiso}}/k_{\text{riso}}$, which occurs when the steady-state concentration of [G] is significant relative to [F] or [E], then eqns. (28)–(31) do not hold, which underscores the need to treat isomerizations as segments with multiple steps and not as a single step as done previously, in addition to treating the chemical conversion and product release as separate steps. Nevertheless, the equations can be used to calculate useful limits for the apparent rate constants of isomerization. When $K_{\text{eq.}(E/F)} = 1$, the equations hold despite the presence of significant [G]; if $K_{\text{eq.}(E/F)} > 1$, then k_{fiso} will be overestimated and k_{riso} underestimated; and if $K_{\text{eq.}(E/F)} < 1$, then k_{fiso} will be underestimated and k_{riso} overestimated. In other words, if an isomerization is detected, the mechanism may be more iso than estimated because of the uncertainty of the presence and relative concentrations of intermediate forms of free enzyme.

In the absence of knowledge about $K_{\text{eq.}(E/F)}$, it is still possible to calculate a quantitative measure of how rate-limiting the isomerization segment is by comparing eqn. (26) to maximal velocities:

$$f_{\text{iso}} = \frac{\frac{1}{k_{\text{fiso}}} + \frac{1}{k_{\text{riso}}}}{\frac{1}{V_f} + \frac{1}{V_r}} \quad (32)$$

The concept of a rate-limiting step has been much abused and lacks an accepted definition (Northrop, 1981; Ray, 1983). Following Johnston (1966) and Boyd (1978), the concept as used here is restricted to reaction processes which are irreversible under initial-velocity conditions at saturating substrate and can be described by a single, apparent first-order rate constant. Because these processes may consist of multiple steps, they are referred to as reaction segments. How rate-limiting each segment is will be proportional to the amount of enzyme participating in it during steady-state turnovers at saturating concentrations of substrates, and equally proportional to the reciprocal of the net rate constant governing the segment, which provides a definition and means of identification and quantification. The fractional reduction of the maximal velocity, represented by f_{iso} , was first introduced as f_v to quantify partially rate-limiting steps from isotope effects (Northrop, 1977). Here it indicates the average impact of the isomerization on the maximal velocities of the reaction in the forward and reverse directions together. The value of f_{iso} will range from zero, meaning an insignificant iso-mechanism, to 1, meaning the isomerization is fully rate-limiting.

Irreversible Iso Uni Uni reactions

Previous discussions of iso-mechanisms have addressed reversible reactions only, perhaps because isomerizations were sought for only in mutases and racemases (Fisher et al., 1986). However, iso-mechanisms have been extended to hydrolytic reactions (Rebholz and Northrop, 1991), which are effectively irreversible because of 55 M water. Rehydration of such enzymes during turnover may itself constitute an 'isomerization' step in their kinetic mechanisms. Irreversibility may result from either an irreversible isomerization segment or an irreversible chemical segment and both conditions reduce to the same general form of the rate equation; however, the definitions of the kinetic constants differ. For an irreversible isomerization segment, the practical relationship is described by eqn. (30). For an irreversible chemical segment, a very different kinetic situation is encountered because no induced transport is possible. On the other hand, the lack of

reversibility in the chemical segment removes the difficulty with traditional product inhibition described by Darvey (1972) and a non-competitive pattern can be obtained in the normal way. This pattern is useful in evaluating iso-mechanisms because the ratio of slope and intercept effects, governed by K_p and K_{tip} respectively, provide a measure of how rate-limiting the isomerization is, due to the relationship:

$$f_{\text{iso}} = \frac{K_p}{K_{\text{tip}}} = \frac{\frac{1}{k_{\text{fiso}}} + \frac{1}{k_{\text{riso}}}}{\frac{1}{V_f}} \quad (33)$$

DISCUSSION

The induced transport experiment of Britton (1973) is a very important contribution to enzyme kinetics because it is the only experimental design which can give unambiguous evidence in support of an iso-mechanism. Detecting the K_{sp} term from the intercept of a non-competitive product inhibition pattern, for example, does not in itself warrant the conclusion that free enzyme exists in two or more forms. Non-competitive terms of a rate equation can result from the formation of a non-productive ternary complex of enzyme, substrate and product (Ray and Roscelli, 1964), or from the presence of two sets of active sites (Kitiakowsky and Rosenberg, 1952). The importance of this design went unnoticed for many years as Britton was unsuccessful in finding a good enzyme candidate for its application. Fisher et al. (1986) recognized this significance and successfully employed Britton's design in a kinetic analysis of proline racemase, and found values for k_{fiso} of approx. 10^5 s^{-1} versus a k_{cat} of $2.6 \times 10^3 \text{ s}^{-1}$. Raines and Knowles (1987) similarly applied the design to triosephosphate isomerase and found values of 10^6 s^{-1} versus $4.3 \times 10^3 \text{ s}^{-1}$ respectively. We (K. L. Rebholz and D. B. Northrop, unpublished work) applied the design to fumarase and found 10^4 s^{-1} versus $1 \times 10^2 \text{ s}^{-1}$, respectively.

In a pair of lengthy and difficult papers, Alberty and Knowles (1987a,b) explored algebraic relationships of Britton's design, and formulated the two roots of α in terms of specific concentrations of substrate, c_D and c_P , termed 'dip switch concentration' and 'peak switch concentration' respectively. Despite the rigour of this system, it has not been applied to iso-mechanisms by other investigators. This may be due to the conceptual difficulty of its formulations, because it employs an unfamiliar nomenclature containing partial equilibrium constants to describe kinetic processes. Also, it is limited to analysis of single-step isomerizations. The accessibility of describing the kinetics of iso-mechanisms in conventional kinetic nomenclature provided by eqns. (25)–(33) has the potential to make Britton's experimental design a much more widely used tool of enzyme kinetics. Moreover, as stated by Raines and Knowles (1987), "catalysis is a cyclic process" requiring enzymes to return to the form which binds substrate; hence, all enzymes are potential candidates for investigation by this experimental design.

Note added in proof (received 11 October 1993)

We have recently shown (K. L. Rebholz and D. B. Northrop, unpublished work) that K_{sp} alone is not a sufficient determinant of competitive inhibition. Cleland's ratio K_{tip}/K_p actually determines whether inhibition contains a competitive component, and Britton's intuition that inhibition will appear competitive in the presence of slow isomerization appears to be correct. This will be the subject of a future publication.

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