Use of Flux Ratio Measurements for the Determination of the Order of Addition of Substrates and Products in Enzyme Reactions

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1. Methods of determining the order of addition of substrates and dissociation of products by using flux ratios are investigated. Where an enzyme obeys hyperbolic steady-state velocity kinetics it is concluded that it may be particularly useful to compare the measured flux ratios with those calculated from the steady-state velocity parameters. 2. An expression is derived relating the relative contribution of the two pathways in a branched pathway to the flux ratios. 3. The relationship of equilibrium-reaction-rate measurements [Boyer & Silverstein (1963) *Acta Chem. Scand.* 17, *Suppl.* 1, S195] to the flux ratios is considered. Equilibrium-reaction rates are shown to be affected both by the addition of substrates and dissociation of products. Methods of analysing the data to distinguish between these events are discussed. 4. Methods of measurement of flux ratios are described, and it is concluded that the non-equilibrium steady-state method is preferable to measurements at chemical equilibrium. 5. The relative significance of flux ratio measurements and steady-state velocity inhibition data is discussed. It is concluded that flux ratios, when taken in conjunction with the inhibition data, provide the least ambiguous information about mechanism.

In a previous paper (Britton, 1966) the kinetics of enzyme reactions were analysed in terms of fluxes, and it was pointed out that certain flux ratios should give information about the mechanism of the addition of substrates and dissociation of products. The present paper develops this concept and in particular considers how the parameters in such measurements may be related to steady-state velocity measurements. Such a comparison is particularly important in the practical case where experimental errors have to be taken into account. Appropriate methods of measurement of these flux ratios are also discussed.

Terminology

The term flux in the following sections is used to describe the rate of conversion of individual molecules from one form into another (Britton, 1966). Thus the flux of A to P (where A is a substrate and P is a product) is the rate of conversion of A molecules into P molecules. If P is present there may also be a flux of P to A. The rate of accumulation of P will be the difference between the fluxes and it is termed the net flux of A to P. At chemical equilibrium the fluxes in the two directions are equal and are synonymous with the equilibrium reaction rate (Boyer & Silverstein, 1963). In general it is necessary to use isotopically labelled substrates to measure fluxes.

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In two-substrate-two-product reactions transfer of a group (X) often occurs and the reaction can be represented as:

Since it will usually be possible to label independently R, R' and X, it should be possible to measure a flux of RX to R and a flux of RX to R'X. Similarly it should be possible to measure a flux of R'X to R' and a flux of R'X to RX. In the following sections the ratios of the fluxes originating from RX and R'X are particularly considered.

Determination of the Order of Addition of Substrates and the Relative Contribution of Branched Pathways

Flux ratio patterns

(a) *Fully ordered mechanisms.* For a fully ordered mechanism (Scheme 1 in Fig. 1) (Britton, 1966) under all conditions, not necessarily those of equilibrium:

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = 1 + \frac{k_{+2}k_{+3}[B]}{k_{-1}(k_{+3} + k_{-2})} \quad (1)$$

and

$$\frac{\text{Flux of Q to B}}{\text{Flux of Q to A}} = 1 + \frac{k_{+2}k_{+3}k_{+4}[B]}{k_{-1}\{k_{+4}(k_{-2} + k_{+3}) + k_{-2}k_{-3}[P]\}} \quad (2)$$

Thus both flux ratios increase linearly from unity with the concentration of B (unless $k_{-1} \rightarrow \infty$), but are

Scheme 1.

Scheme 2.

Scheme 3.

Fig. 1. Schematic diagrams of enzyme mechanisms

Steps at equilibrium are indicated by double-headed arrows. k_{+1} , k_{-1} etc. are rate constants, and K_P , K'_P etc. are equilibrium constants. $K_{PQ} = K'_Q K_P = K'_P K_Q$. For the pyruvate kinase reaction, A = phosphoenolpyruvate, B = ADP, P = ATP and Q = pyruvate (Dann & Britton, 1978). In Schemes 2 and 3, the substrate and product molecules have been omitted for simplicity.

Fig. 2. Flux ratio patterns

The numbers adjacent to the lines refer to the Schemes in Fig. 1. For the ordered reaction represented by Scheme 1, the ratio Flux of P to B/Flux of P to A (a) increases linearly with the concentration of B from unity when $[B] = 0$. The reciprocal of the ratio (b) is independent of the concentration of A. This ratio is unity as indicated if $[B] = 0$. It will be less than unity if B is present. For the random reaction, Scheme 2, the ratio Flux of P to B/Flux of P to A (a) increases hyperbolically with the concentration of B. If $[A] = 0$ the initial flux ratio is unity as indicated, and the ratio rises to the plateau value R_B (eqn. 9). If A is present, the initial ratio will be less than unity and the plateau value will be proportionately lower. Similar considerations apply to the reciprocal of this ratio (b). For the random reaction, Scheme 3, the ratio Flux of P to B/Flux of P to A (a) increases hyperbolically with the concentration of B from unity to a plateau R_B (eqn. 9). The reciprocal of the ratio (b) is independent of the concentration of A. It is unity, as indicated, if $[B] = 0$. It will be less than unity if B is present.

unaffected by the concentration of A (lines 1 in Figs. 2a and 2b). The concentration of the product Q, which is involved in the flux ratio, does not appear in the expressions, but the concentration of the other product P may appear (eqn. 2). Scheme 1 gives hyperbolic steady-state velocity kinetics, and the coefficients in eqns. (1) and (2) can be quantitatively derived from the appropriate Dalziel coefficients (Dalziel, 1957) as described below.

(b) *Random and partly ordered mechanisms.* For the general mechanism with a random order of addition of substrates and dissociation of products (Scheme 2 in Fig. 1) under all conditions (Britton, 1966):

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = \frac{1 + \alpha[B]}{1 + \beta[B]} \frac{1 + \gamma[A]}{1 + \delta[A]} \quad (3)$$

where

$$\alpha = \frac{k_{+2}(X + k_{-7})}{k_{-1}(X + k_{-2} + k_{-7})} \quad (4)$$

$$\beta = \frac{k_{+2}k_{-7}}{k_{-1}(k_{-2} + k_{-7})} \quad (5)$$

$$\gamma = \frac{k_{+7}(X + k_{-2})}{k_{-6}(X + k_{-2} + k_{-7})} \quad (6)$$

$$\delta = \frac{k_{+7}k_{-2}}{k_{-6}(k_{-2} + k_{-7})} \quad (7)$$

and

$$X = \frac{k_{+3}\{[k_{+4}k_{+5}/(k_{+9} + k_{-8}[Q])] + k_{+4}\}}{\{k_{-3} + k_{+8} + k_{+4}k_{+5}/(k_{+5} + k_{-4}[Q])\}} \quad (8)$$

$[X(\text{EAB})]$, where (EAB) denotes the amount EAB, represents the flux of EAB to P. The expression for the ratio Flux Q to B/Flux Q to A is the same as eqn. (3) apart from the expression for X , where the rate constants for the two pathways for dissociation of product are transposed.

As in the ordered mechanism the concentration of the product P that is involved in the flux ratio (eqn. 3) does not appear in the expressions. In contrast with the ordered addition of substrates, from eqn. (3), the ratio Flux of P to B/Flux of P to A rises hyperbolically to a plateau as the concentration of B is increased (line 2 in Fig. 2a). When the concentration of A is zero (or if the concentration of A does not affect the flux ratio; see below) the value for the flux ratio at $[B] = 0$ will be unity, and at high concentrations of B the flux ratio reaches a plateau R_B given by:

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = R_B = \left(\frac{k_{-2} + k_{-7}}{k_{-7}} \right) \left(\frac{X + k_{-7}}{X + k_{-2} + k_{-7}} \right) \quad (9)$$

The ratio $k_{-7}/(k_{-2} + k_{-7})$ represents the number of EAB molecules that dissociate into EB expressed as a

fraction of the number that dissociate into either EA or EB. From eqn. (9):

$$\frac{k_{-7}}{k_{-2} + k_{-7}} \leq \frac{1}{R_B} \quad (10)$$

The equality applies if X is very large.

Similarly the ratio Flux of P to A/Flux of P to B rises hyperbolically with the concentration of A to a plateau R_A (line 2 in Fig. 2b) and:

$$\frac{k_{-2}}{k_{-2} + k_{-7}} \leq \frac{1}{R_A} \quad (11)$$

From eqns. (10) and (11), it follows that:

$$R_B < R_A/(R_A - 1) \quad (12)$$

If $k_{+8} = k_{-8} = k_{+9} = k_{-9} = 0$ in Scheme 2 in Fig. 1, the dissociation of products is ordered. This influences the value of X in eqns. (3)–(12), but does not affect the conclusion that a branched addition of substrates gives a flux ratio that varies hyperbolically with the concentrations of the substrates. Conversely an ordered addition of substrates will give a flux ratio that is independent of one substrate and varies linearly with the other as with the fully ordered mechanism (Scheme 1 in Fig. 1), even if the dissociation of products is random. If the dissociation of the products in this latter case is rapid (k_{+5} and $k_{+9} \rightarrow \infty$), hyperbolic steady-state velocity kinetics will be obeyed and the slope of the line (line 1 in Fig. 2a) can be derived from the Dalziel coefficients. If the dissociation is not rapid, hyperbolic steady-state velocity kinetics will not be obeyed with respect to the products, but the slope can still be predicted from appropriate flux measurements (see below).

If the dissociation of B from EB is rapid compared with other steps in the reaction (Scheme 3 in Fig. 1) or if $k_{-7}/(k_{-2} + k_{-7})$ is very small, then the flux ratio will be independent of A but will vary hyperbolically with the concentration of B (lines 3 in Figs. 2a and 2b). This pattern is particularly relevant to the behaviour of rabbit muscle pyruvate kinase (Dann & Britton, 1978).

A rapid random addition of substrates ($k_{+1}, k_{-1}, k_{+6}, k_{-6} \rightarrow \infty$) will give a flux ratio that is unity under all conditions.

Determination of the relative contribution of the two pathways in a branched mechanism from the flux ratios

In the absence of substrates, with the reaction proceeding in the reverse reaction, the number of EAB molecules dissociating to E via EA expressed as a fraction of the number of EAB molecules dissociating via either EA or EB is $k_{-2}/(k_{-2} + k_{-7})$, and the proportion dissociating along the alternative pathway via EB is $k_{-7}/(k_{-2} + k_{-7})$. The maximum contributions of the two pathways to the enzyme reaction in the absence of substrates are therefore $1/R_A$ and $1/R_B$ (eqns. 10 and 11).

In the presence of substrates the net flux of E to EAB via EA (Scheme 2 in Fig. 1) is given by the expression (Britton, 1966):

$$\left[\frac{k_{+1}k_{+2}[A][B](E)}{k_{-1}+k_{+2}[B]} \right] - \left[\frac{k_{-1}k_{-2}(EAB)}{k_{-1}+k_{+2}[B]} \right] \quad (13)$$

where (E) and (EAB) are the amounts of E and EAB respectively. The parts in large square brackets refer to the fluxes of E to EAB and of EAB to E. Correspondingly the net flux from E to EAB via EB is given by the expression:

$$\left[\frac{k_{+6}k_{+7}[A][B](E)}{k_{-6}+k_{+7}[A]} \right] - \left[\frac{k_{-6}k_{-7}(EAB)}{k_{-6}+k_{+7}[A]} \right] \quad (14)$$

Let R_F be the proportion of the net flux proceeding via EB; then:

$$R_F = \frac{\text{Net flux from E to EAB via EB}}{\text{Total net flux from E to EAB via EA and EB}} \quad (15)$$

From eqns. (3)–(8), (14) and (15), and since $k_{+1}k_{+2}k_{-6}k_{-7} = k_{+6}k_{+7}k_{-1}k_{-2}$:

$$R_F = \frac{k_{-7}}{(k_{-2}+k_{-7})(1+\delta[A]+\beta[B])} + \frac{\beta[B]}{(1+\delta[A]+\beta[B])} \quad (16)$$

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = 1 + \frac{(k_{+4}+k_{+8})k_{+2}k_{+3}[B]}{k_{-1}[k_{+3}(k_{+4}+k_{+8})+k_{-2}(k_{-3}+k_{+4}+k_{+8})(1+Y[P])]} \quad (21)$$

where:

$$Y = \frac{k_{-2}k_{-4}k_{-3}k_{+4}}{k_{+5}(k_{+4}+k_{+8})[(k_{+3}+k_{-2})(k_{+4}+k_{+8})+k_{-2}k_{-3}] \left(1 + \frac{k_{+8}k_{-4}[Q]}{k_{+5}(k_{+4}+k_{+8})} \right)} \quad (22)$$

and from eqns. (10) and (12):

$$R_F = \frac{1}{1+\delta[A]+\beta[B]} \left(\beta[B] + \left[\leq \frac{1}{R_B} \geq \frac{R_A-1}{R_A} \right] \right) \quad (17)$$

Eqn. (17) enables limits to be set on the total contribution of the EB pathway. It applies whether there is net forward or backward reaction.

Correlation of parameters derived from flux ratios with those determined from steady-state velocities

(a) *Ordered addition of substrates.* The velocity v of an ordered enzyme reaction (Scheme 1 in Fig. 1) in the presence of product P is given by the expression:

$$\frac{1}{v} = \frac{1}{(E_T)} \left(\phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} + \phi^P[P] + \frac{\phi_B^P[P]}{[B]} + \frac{\phi_{AB}^P[P]}{[A][B]} \right) \quad (18)$$

where (E_T) is the total amount of enzyme and ϕ_0 etc. are parameters depending on the rate constants (Dalziel, 1957).

For Scheme 1 in Fig. 1, from eqn. (1):

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = 1 + \frac{\phi_A[B]}{\phi_{AB}} \quad (19)$$

and from eqn. (2):

$$\frac{\text{Flux of Q to B}}{\text{Flux of Q to A}} = 1 + \frac{\phi_A[B]}{\phi_{AB} + \phi_{AB}^P[P]} \quad (20)$$

Similarly for a rapid random dissociation (Scheme 2; $k_{+6} = k_{-6} = k_{+7} = k_{-7} = 0$; $k_{+5}, k_{+9} \rightarrow \infty$) eqn. (18) also applies with $\phi^P = \phi_B^P = 0$. Both flux ratios are then given by eqn. (19). Thus, if the mode of dissociation of products is known, the coefficients in the flux ratio equations can be obtained directly from parameters of the steady-state velocity equation.

If the addition of substrates is ordered but the mode of dissociation is not known, the following general procedure may be used to determine the coefficients in the flux ratio equations. It is applicable even if the inhibition patterns are non-linear. An ordered addition of substrates and a random dissociation of products is obtained in Scheme 2 in Fig. 1 if $k_{+6} = k_{-6} = k_{+7} = k_{-7} = 0$. Then from eqns. (3)–(8):

and from the steady-state rate equation for this mechanism:

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = 1 + \frac{\phi_A[B]}{\phi_{AB}(1+Y[Q])} \quad (23)$$

Similarly, considering the reaction in the forward direction:

$$\frac{\text{Flux of A to P}}{\text{Flux of A to Q}} = 1 + \frac{Y[Q]}{\{1 + (\phi_A/\phi_{AB})[B]\}} \quad (24)$$

Thus, if the ratio Flux of A to P/Flux of A to Q is measured and the Dalziel coefficients ϕ_A and ϕ_{AB} are known, Y can be determined. Insertion of the value of Y into eqn. (23) then enables the theoretical values for the ratio Flux of P to B/Flux of P to A to be calculated. Since Y contains a term involving the concentration of Q, both flux ratio measurements should be made at the same concentration of Q. Alternatively, if the ratio Flux of B to P/Flux of B to

Q is measured rather than the ratio Flux of A to P/Flux of A to Q, then:

$$\frac{\text{Flux of B to P}}{\text{Flux of B to Q}} = 1 + Y[Q] \quad (25)$$

Y in this case can be calculated directly from the flux ratio.

(b) *Random addition of substrates.* In general a mechanism with a random addition of substrates will not give hyperbolic steady-state velocity kinetics unless the addition is rapid and random (Scheme 2 in Fig. 1; $k_{+1}, k_{-1}, k_{+5}, k_{-5} \rightarrow \infty$). In the latter case the flux ratios will be unity under all conditions, as discussed above. However, it is possible for non-rapid random mechanisms to approximate to hyperbolic steady-state velocity kinetics. Thus the data in the following paper (Dann & Britton, 1978) indicate the branched mechanism (Scheme 3 in Fig. 1). This mechanism approximates to hyperbolic steady-state velocity kinetics under appropriate conditions and from eqn. (1) (Appendix I to Dann & Britton, 1978):

$$\phi_A/\phi_{AB} = \frac{k_{+2}(k_{-7} + k_{+3})}{k_{-1}(k_{-2} + k_{-7} + k_{+3})} + \frac{1}{K_B} \quad (26)$$

Also, from eqn. (3), since $X = k_{+3}$ and $\gamma = \delta = 0$:

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = \frac{1 + \alpha[B]}{1 + \beta[B]} = \frac{1 + \{k_{+2}(k_{+3} + k_{-7})[B]/k_{-1}(k_{-2} + k_{-7} + k_{+3})\}}{1 + \{k_{+2}k_{-7}[B]/k_{-1}(k_{-2} + k_{-7})\}} \quad (27)$$

Comparison of eqns. (26) and (27) shows that the coefficient α in the numerator of the flux ratio equation is equal to $(\phi_A/\phi_{AB}) - (1/K_B)$ and can therefore be calculated from the Dalziel coefficients. From this value and the measured flux ratio, the coefficient in the denominator (β) can be derived.

Determination of the Order of Dissociation of the Products

Flux ratio measurements of the type just discussed may be used to examine the order of dissociation of the products if the reaction is considered in the forward rather than in the reverse direction. For an ordered reaction (Scheme 1 in Fig. 1) the flux ratios are related to the Dalziel coefficients as follows:

$$\frac{\text{Flux of A to P}}{\text{Flux of A to Q}} = 1 + \frac{\phi_{AB}^P[P]}{\phi_{AB} + \phi_A[B]} \quad (28)$$

and

$$\frac{\text{Flux of B to P}}{\text{Flux of B to Q}} = 1 + \frac{\phi_{AB}^P[P]}{\phi_{AB}} \quad (29)$$

If the addition of the substrates A and B is rapid and random, the ratios Flux of A to P/Flux of A to Q and

Flux of B to P/Flux of B to Q are equal and are given by eqn. (29). Where the addition is random but not rapid as in Scheme 3 in Fig. 1, the ratio Flux of A to P/Flux of A to Q is intermediate in value between that given by eqns. (28) and (29) (Dann & Britton, 1978).

Isomerization of Binary Complexes: Isomerization of the Free Enzyme

The above equations still apply even if the formation of the binary complexes with substrates or products involves a series of isomerizing steps of the type:

or if there is an isomerization of the free enzyme of the type:

Flux Ratios for a Ping-Pong Mechanism

Consider the general mechanism:

where $A = RX$, $B = R'$, $Q = R$ and $P = R'X$, and steps at equilibrium are represented by double-headed arrows. This mechanism contains all the steps that should be recognizable by steady-state kinetics. The steady-state velocity v for this scheme is given by the expression:

$$\frac{1}{v} = \frac{1}{(E_T)} \left(\phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \phi^Q[Q] + \frac{\phi_A^Q[Q]}{[A]} + \frac{\phi_B^Q[Q]}{[B]} \right) \quad (30)$$

where (E_T) represents the total quantity of enzyme. The expressions for flux ratios in terms of rate constants have been given elsewhere (Britton, 1966), and are related to the ϕ values in eqn. (30) as follows:

$$\frac{\text{Flux of P to B}}{\text{Flux of P to A}} = 1 + \frac{\phi^Q[B]}{\phi_B^Q} + \frac{\phi_A[B]}{\phi_{AB}^Q[Q]} \quad (31)$$

and

$$\frac{\text{Flux of A to Q}}{\text{Flux of A to P}} = 1 + \frac{[Q]}{\phi_A} \left(\phi_A^Q + \frac{\phi_{AB}^Q}{[B]} \right) \quad (32)$$

As in the ordered sequential mechanism, therefore, the ratio Flux of P to B/Flux of P to A increases linearly with the concentration of B but is unaffected by the concentration of A. However, unlike the ordered sequential mechanism, the ratio rises towards infinity at low concentration of Q. This corresponds to the exchange between P and B that the mechanism catalyses in the absence of Q and A.

Ratio Flux of B to P/Flux of A to Q

Boyer & Silverstein (1963) proposed measurements of equilibrium reaction rates between separate pairs of substrates as a test of enzyme mechanism. They showed that, for an ordered mechanism with A adding to the enzyme before B, and P dissociating first from the ternary complex, exchange between A and Q should fall to zero as the concentrations of B and P are increased (while maintaining the relative concentrations of B and P so that the equilibrium was not disturbed), whereas exchange between B and P should rise to a maximum. In contrast, if both A and B, and P and Q, add in random order, neither the exchange between B and P or A and Q fall to zero as the concentrations of B and P are increased. However, there is an ambiguity in interpreting such measurements in that it is only necessary for the addition of one pair of substrates to be ordered for exchange between B and P to fall to zero (Boyer, 1959).

The relationship between equilibrium reaction rates and the flux ratios may be demonstrated as follows. The reaction rate at equilibrium is identical with the flux at chemical equilibrium, and therefore the ratio of the equilibrium reaction rates between B and P and A and Q is given by the ratio of the fluxes. Further:

$$\frac{\text{Flux of B to P}}{\text{Flux of A to Q}} = \left(\frac{\text{Flux of B to P}}{\text{Flux of A to P}} \right) \left(\frac{\text{Flux of A to P}}{\text{Flux of A to Q}} \right) \quad (33)$$

The forward and reverse fluxes are equal at equilibrium and therefore:

$$\frac{\text{Flux of B to P}}{\text{Flux of A to Q}} = \left(\frac{\text{Flux of P to B}}{\text{Flux of P to A}} \right) \left(\frac{\text{Flux of A to P}}{\text{Flux of A to Q}} \right) \quad (34)$$

From eqn. (34) it follows that the ratio of the equilibrium reaction rates between B and P, and A and Q, is equal to the product of the flux ratios just discussed. This demonstrates how the equilibrium rate measurements are affected by events on both sides of the ternary complex. It also suggests how those events may be distinguished. Thus, if the association of substrates and dissociation of products is ordered (Scheme 1 in Fig. 1), from eqns. (19) and (28):

$$\frac{\text{Flux of B to P}}{\text{Flux of A to Q}} = 1 + \frac{\phi_A[B]}{\phi_{AB}} + \frac{\phi_{AB}^P[P]}{\phi_{AB}} \quad (35)$$

The ratio of the fluxes varies linearly with [B] or [P] and the slopes are given by the Dalziel coefficients. If the concentrations of the pair B and P are increased the slope will be the sum of both terms in eqn. (35), whereas if the pairs B and Q or A and P are increased the slopes are given by ϕ_A/ϕ_{AB} and ϕ_{AB}^P/ϕ_{AB} respectively. In contrast, if the addition of substrates is

ordered but the dissociation of the products is rapid and random, from eqns. (19) and (28):

$$\frac{\text{Flux of B to P}}{\text{Flux of A to Q}} = 1 + \frac{\phi_A[B]}{\phi_{AB}} \quad (36)$$

Thus in this case the slope is given by ϕ_A/ϕ_{AB} when the concentrations of B and P are increased; the slope is the same if the concentrations of A and P are increased but is zero if those of B and Q are increased. Corresponding conclusions apply if the addition of substrates should be rapid and random but the dissociation ordered.

Should the addition of substrates or products or both be random but not rapid, the ratio of the equilibrium fluxes will not increase linearly with substrate concentrations but will tend to a plateau. Suppose that the addition of substrates is completely ordered, and that the dissociation of products although random is relatively ordered. Under these conditions the ratio Flux of A to P/Flux of A to Q may reach a fairly high value at high concentrations of P. From eqn. (12) therefore the concentration of Q will have relatively little influence on this ratio. Thus, from eqn. (34), when the concentrations of A and P are increased the ratio of the equilibrium reaction rates will increase rapidly but non-linearly, whereas if the concentrations of A and Q are increased the increase, although much less rapid, will be very nearly linear. A substantially non-linear increase in the latter case would indicate that the addition of substrates must also be random.

In most cases measurement of the ratio Flux of B to P/Flux of A to Q will only be required to avoid the use of tritium or deuterium as with the dehydrogenases (see the Discussion section). Measurement of flux ratios of the type Flux of A to P/Flux of A to Q by the non-equilibrium method requires the use of only one radioactive isotope at a time. The dependence of this ratio on one of the products (P or Q) therefore will normally be measurable without the use of isotopic hydrogen. As discussed elsewhere (Britton, 1977), in such cases a combination of non-equilibrium measurements with equilibrium measurements of the ratio Flux of B to P/Flux of A to Q may be a more effective way of determining the mechanism than equilibrium measurements alone.

Measurement of flux ratios

In principle, the Flux of A to P can be determined by adding labelled substrate A and measuring the initial rate of appearance of label in P. The initial rate of appearance of the label in P depends only on the Flux of A to P. Correspondingly the ratio Flux of A to P/Flux of A to Q can be determined with doubly labelled substrate. There is no requirement for chemical equilibrium and the measurements can be

made with any combination of concentrations of substrates and products. In practice, however, it is convenient to make the measurements either (a) under non-equilibrium conditions with the reaction proceeding in the forward direction in the absence of one of the products or (b) at equilibrium.

Non-equilibrium measurements

To measure the ratio Flux of A to P/Flux of A to Q as a function of the concentration of P, the substrates are incubated with the enzyme in the presence of labelled P in known concentration but in the absence of Q. The initial rate of appearance of label in A is measured and gives the flux of P to A. The rate of the chemical reaction is also determined. This gives the Flux of A to Q, since in the absence of Q there is no flux of Q to A. The net fluxes of A to P and of A to Q must be the same and therefore:

$$\begin{aligned}\text{Net flux of A to Q} \\ &= \text{Flux of A to Q} \\ &= (\text{Flux of A to P}) - (\text{Flux of P to A})\end{aligned}$$

hence

$$\frac{\text{Flux of A to P}}{\text{Flux of A to Q}} = 1 + \frac{\text{Flux of P to A}}{\text{Net flux of A to Q}} \quad (37)$$

Thus the ratio Flux of A to P/Flux of A to Q is given by the initial rate of appearance of label in A and by the initial rate of the chemical reaction. By altering the concentration of P, the dependence of the ratio on the concentration of P can be determined. Alternatively, the measured appearance of label in A in a single experiment can be compared with the calculated quantity as a function of the amount of chemical reaction if it is assumed how the flux ratio depends on the concentration of P (Dann & Britton, 1978). Corresponding measurements may be made with Q in the absence of P. Qualitative measurements of this type were reported by Zatman *et al.* (1953) with yeast NAD⁺ nucleosidase and with hexokinase by Kosow & Rose (1970), although the data were not analysed in terms of fluxes.

Equilibrium measurements

To determine the ratio Flux of A to P/Flux of A to Q at chemical equilibrium, as a function of the concentration of P or Q, it may be noted that the ratio is unaffected by the concentration of A whatever the mechanism (eqns. 1, 2 and 3) unless A affects the enzyme allosterically. Such allosteric changes, however, will only be important if they lead to a change in mechanism or to a change in the relative rates of the different steps. A change in the proportion of active to inactive enzyme will not influence the ratio. With this proviso, the concentration of A

may be adjusted to maintain chemical equilibrium at all times. A may be doubly labelled, so that the isotopic labels appear in both P and Q, but in some cases this may be technically difficult because of an unfavourable equilibrium constant. In such cases P and/or Q may be appropriately labelled and the rate of transfer of label to A measured, since at equilibrium the fluxes in the two directions are equal.

The non-equilibrium method of measuring flux ratios is particularly useful because it measures directly the deviation of the ratio from unity. Nevertheless it may be necessary to use the equilibrium method, particularly if the equilibrium constant is unfavourable. Thus with kinase enzymes the equilibrium usually lies far to the right. To study the addition of substrates by the non-equilibrium method the reaction must be allowed to occur in the reverse direction. However, the amount of reaction that can be allowed to occur is small. This limits the amount of incorporation of label into the product and thus the sensitivity of the method. The addition of any enzyme to remove one of the substrates as soon as it is formed may be particularly useful in such a case.

Discussion

The conventional steady-state method of determining the order of addition of substrates and dissociation of products depends on the examination of the patterns of inhibition produced by the products or by analogues of the products or substrates (Cleland, 1963*a,b*). It has yielded important information about the mechanisms of many enzymes, although it gives no indication of the relative contributions of alternative pathways. However, it is an extrapolation technique and experimental errors, or a failure of the enzyme to conform to hyperbolic kinetics (as may be the case with a branched pathway), may have a large effect on the point of intersection of the reciprocal plots and consequently on the conclusions that are drawn. This is particularly important, for example, when the non-competitive component in inhibition by a product is small. Further, the formation of dead-end complexes may interfere with the interpretation. In consequence, results obtained in inhibition experiments may be inconsistent, as in the following paper (Dann & Britton, 1978), where the pyruvate and ATP inhibition data might suggest an ordered reaction with pyruvate dissociating first from the ternary complex.

Measurement of the ratio Flux of Q to B/Flux of Q to A or Flux of P to B/Flux of P to A with isotopic tracers gives direct evidence about the addition of substrates to the enzyme and it is free from some of the restrictions that apply to conventional steady-state velocity measurements. Thus it is not a necessary requirement that the enzymes obey hyperbolic steady-state velocity kinetics, and neither the presence

of isoenzymes with different K_m values nor the formation of dead-end complexes needs interfere. The demonstration of a ratio greater than unity is unequivocal evidence that an appreciable part of the reaction involves the addition of A first to the enzyme in a rate-limiting step. Similarly, if the ratio Flux of P to A/Flux of P to B is greater than unity, an appreciable part of the reaction must involve the addition of B to the enzyme in a rate-limiting step. If plateau values (R_A and R_B , eqns. 10 and 11) can be found, limits can be set on the contributions of the two pathways.

If the addition of A and B is ordered with A adding first to the free enzyme, the ratio Flux of P to B/Flux of P to A increases linearly from unity with the concentration of B, but the ratio is unaffected by the concentration of A. This has to be distinguished from the pattern obtained for a random addition of A and B, where $k_{-6} \rightarrow \infty$ or $k_{-7}/(k_{-2}+k_{-7})$ is very small (Scheme 2 in Fig. 1). Under these latter conditions the flux ratio is unaffected by the concentration of A but it increases non-linearly towards a plateau with the concentration of B. If the curvature of the plot should be small, it may be difficult to distinguish this latter mechanism from an ordered addition. However, if the flux ratio reaches a relatively high value, for example 10 (or extrapolates to such a value), it must follow that at low concentrations of B at least 90% of the reaction follows an ordered pathway (eqn. 17). The possible contribution of the alternative pathway at higher concentrations of B can also be estimated. If the maximum flux ratio is found to be relatively small and if the enzyme approximates to hyperbolic steady-state velocity kinetics, it is valuable to compare the measured flux ratio with those calculated for an ordered addition. The ϕ values will be affected by the presence of a branched pathway in a manner that does not depend on the contribution of the alternative pathway to the overall reaction. For this reason the measured value of the flux ratio will usually be very much less than the calculated value in the presence of an alternative pathway, even if this pathway makes only a small contribution to the overall reaction, as found in the following paper on rabbit muscle pyruvate kinase (Dann & Britton, 1978).

When a flux ratio of unity is found, it is of particular importance to compare the experimental value with that calculated from the steady-state velocity kinetics for an ordered addition of substrates or dissociation of products. Thus, for example, consider the ratio Flux of A to P/Flux of A to Q. This ratio will be unity under all conditions (1) if there is an ordered dissociation of products with $k_{+4} \rightarrow \infty$ (Scheme 1 in Fig. 1), (2) if there is a random dissociation of products with $k_{+5}, k_{+9} \rightarrow \infty$ (Scheme 2 in Fig. 1), (3) if there is a random dissociation of products with $k_{+3} \rightarrow \infty$ and $k_{+8}/(k_{+4}+k_{+8}) \rightarrow 0$, and (4) if there is a random dissociation of products with $k_{+9} \rightarrow \infty$ and

$k_{+4}/(k_{+4}+k_{+8}) \rightarrow 0$. If the dissociation of products is random, it is very unlikely that the flux ratio calculated for an ordered dissociation from the steady-state velocity parameters will give a value of unity (it would only arise if there were infinite co-operativity in the binding of products along one pathway), and indeed the difference may be gross, as in the following paper (Dann & Britton, 1978). The random dissociations represented by (2)–(4) above are closely related and cannot be distinguished by the flux ratios. However, if in (3) $k_{+8} > k_{+9}$ or in (4) $k_{+4} > k_{+5}$, the enzyme will not obey hyperbolic steady-state velocity kinetics.

The measurement of the fluxes between separate pairs of substrates, for example between B and P and A and Q (Boyer & Silverstein, 1963), has the disadvantage that the measured values are affected by events on both sides of the central complex. The measurements must also be made at equilibrium. Nevertheless, with the dehydrogenases for example, such measurements avoid the use of deuterium or tritium, which may show serious isotope effects. It will usually be most appropriate to combine such measurements with measurements of the flux ratios under non-equilibrium conditions (Britton, 1977).

The most effective way of measuring the ratio Flux of A to P/Flux of A to Q appears to be to measure the incorporation of label from one of the products into A while the reaction is proceeding in the forward direction and in the absence of the other product. Not only is the deviation of the flux ratio from unity measured directly, but it will often be possible, by using the integrated equation, to examine the behaviour of the flux ratio over a range of concentrations in a single experiment (Dann & Britton, 1978). Clearly the same procedure may be used to measure ratios of the type Flux of P to A/Flux of P to B, and the equilibrium method would only seem appropriate when the non-equilibrium method is impractical for technical reasons. In the non-equilibrium method it is important that only one product should be present in significant quantity. In the general case when both products are present the incorporation of label into a substrate from a product depends on the ratio of the forward to the reverse fluxes (Britton, 1966, 1973).

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