Isomerization of the free enzyme versus induced fit: effects of steps involving induced fit that bypass enzyme isomerization on flux ratios and countertransport

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In a single-substrate–single-product enzyme reaction, ‘countertransport’, which indicates that the ratio of the forward to the reverse fluxes is less than that expected from the Independence Relationship, is regarded as strong evidence for the free enzyme existing in two states, one of which combines with the substrate and the other with the product, with a slow isomerization between the two conditions. To account for positive and negative co-operativity, found with some enzymes, additional induced-fit reactions bypassing at least part of the isomerization have been proposed. The effects of such additional steps have been examined, using two models: in one, (a), the enzyme passes through an intermediate state during its isomerization, and both substrate and product may react with this state to give rise to the binary complexes; in the other, (b), the substrate may react with the enzyme as soon as the product is released and similarly with the reverse reaction, the isomerization thereby being bypassed completely. In the presence of such additional steps, the following can be concluded. (i) The data should be analysed in terms of the flux ratios, rather than observation of the amount of countertransport. (ii) The additional bypassing steps markedly change the pattern of dependence of the flux ratio on substrate and product concentrations. At high substrate and product concentrations, the ratio remains very dependent on how far the reaction is from equilibrium, and the kinetics are asymmetric. (iii) The mechanism causing the flux ratio to be less than that

given by the Independence Relationship differs from that previously described, in that, at least in part, it arises from a 1:1 exchange between substrate and product. (iv) Despite this novel mechanism, there must be two states of the enzyme, combining respectively with substrate and product, and these must not be in rapid exchange. Thus countertransport remains very strong evidence for the existence of two such states. It is no longer a requirement that the enzyme states should be linked by an isomerization step. (v) Under no conditions can the flux ratio exceed that given by the Independence Relationship. (vi) Under unusual conditions the isomerization of the enzyme in model (b) may be undetectable by steady-state kinetics. (vii) Measurements of the coefficients in the flux ratio equations enable limits to be set to certain ratios of the rate constants. In addition to these conclusions, methods are described for (viii) analysing flux ratio data for the presence of induced fit steps and (ix) determining flux ratios from induced transport curves. The derivation of steady state–velocity equations show that: (x) both models may give rise to positive and negative ‘co-operativity’ and sigmoid substrate–velocity curves, but that, under conditions giving rise to sigmoid curves, the deviation of the flux ratio from that required by the Independence Relationship may be difficult to demonstrate because of the asymmetry of the system. Under all conditions the fluxes at equilibrium should obey hyperbolic kinetics.

INTRODUCTION

The demonstration that kinetic concepts taken from the field of permeability could be applied to the study of enzyme reactions [1–9] introduced a number of new techniques to enzymology. The concept of flux is central to these techniques and refers to the rate of conversion of individual molecules of substrate into product, and vice versa. The rate of chemical accumulation of product is determined by the difference between the two fluxes and is termed the net flux.

If the conversions of molecules of substrate to product and vice versa occur independently, in the sense that there is no transfer of free energy between individual molecules, the ratio of the forward to reverse fluxes must obey the Independence Relationship [2]. For a single-substrate–single-product reaction, the relationship is:

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = \frac{S}{PK} \quad (1)$$

where S and P refer to the chemical activities of the substrate and product and K is the equilibrium constant for the reaction ($K = S_{\text{eq}}/P_{\text{eq}}$, where S_{eq} and P_{eq} are equilibrium values). Activities appear in the expression because of the thermodynamic derivation of the relationship. In dilute solution, concentrations may be substituted for activities, particularly if any ionic effects of the substrate or product are swamped by buffer.

According to eqn. (1), at equilibrium, the flux ratio should be unity. Clearly, this must be the case whatever the mechanism of the reaction, and the significance of the equation is trivial. However, if an excess of substrate is present and there is a net reaction, then it is possible for either:

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} < \frac{S}{PK} \quad (2)$$

or

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} > \frac{S}{PK} \quad (3)$$

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The inequality shown in eqn. (2) can only occur if there is a mechanism in the reaction pathway that enables the free energy released when a molecule of substrate passes down its free energy gradient during its conversion to product to be transferred to another molecule to facilitate the reverse reaction. In contrast, the inequality in eqn. (3) requires that the reaction mechanism must transfer free energy derived from the conversion of substrate into product to other reactant molecules so that the forward reaction is facilitated.

Britton [2,4] suggested that for a single-substrate–single-product reaction, if the inequality shown in eqn. (2) is observed and the enzyme has a single active centre, it must be strong evidence that, when the product is released, the enzyme is in a different conformation from that which combines with the substrate and that the isomerization of the enzyme from one form to the other is slow. For an enzyme with more than one active centre, however, the inequality might arise from an interaction between active centres, although the interaction required would be of a rather unlikely type. In contrast, if the inequality in eqn. (3) is observed, Britton [2,4,5] suggested that one mechanism might involve several molecules of the substrate binding to the enzyme as part of a sequence of steps leading to the formation of product: alternatively [5], there may be co-operation between active centres in a multicentric enzyme, and, when a molecule of substrate is converted into product at one active centre, the reaction is facilitated in the same direction at another centre.

In a single-substrate–single-product reaction, an induced-transport experiment may be carried out by incubating isotopically labelled substrate with the enzyme until equilibrium has been reached and adding unlabelled substrate. If the Independence Relationship is obeyed [eqn.(1)] during the subsequent reaction, the distribution of label (total not specific radioactivity) between substrate and product does not change. If there is a transfer of free energy of the type referred to in relation to eqn. (2), countertransport occurs with a temporary increase in the amount of label in the substrate. If the transfer of free energy is as described in relation to eqn. (3), cotransport occurs with a temporary increase in the amount of label in the product. Variations of this test are possible with, for example, all of the label initially in either the product or substrate [8]. In all such experiments, not only is the direction of transport determined by the direction of the inequalities in eqns. (2) and (3), but the amount of co- or counter-transport is solely determined by the magnitude of any deviation from the Independence Relationship.

Britton and co-workers [6,8–15] applied these techniques to a study of the phosphoglucomutase and phosphoglyceromutase enzymes. Apart from wheatgerm phosphoglyceromutase, cotransport was observed using ^{32}P -labelled substrates of a degree appropriate for a phosphoenzyme mechanism, and which enabled alternative mechanisms to be excluded. With wheatgerm phosphoglyceromutase, a direct transfer of the phosphoryl group was demonstrated. Enzyme isomerization was examined using ^{14}C -labelled substrates, but no countertransport was observed apart from a very small amount with rabbit muscle phosphoglucomutase at very high substrate concentrations [10]. Even with the rabbit muscle phosphoglucomutase therefore any isomerization step must be extremely rapid.

Countertransport is now considered very strong evidence for enzyme isomerization as discussed by Rebholz and Northrop [16]. Certain criticisms by these authors of the detailed kinetic analysis of Britton [8] arose from misunderstandings about the

need to maintain the Michaelis constants at a constant value while the effects of an isomerizing step are being considered and the need to limit the number of steps in a reaction to only those that can be detected by steady-state analysis (see refs. [9] and [17]). The phenomenon of countertransport has been observed with proline racemase [18] and with triose phosphate isomerase [19]. More recently, marked countertransport has been found with fumarase-C [20]. This enzyme shows negative co-operativity in that the V_{max} of the enzyme is considerably greater than would have been expected from extrapolation from the velocities at lower substrate concentrations. Rose et al. [20] therefore suggest that the enzyme, in isomerizing from the form that binds the product to the form that binds the substrate, passes through an intermediate state that can bind either substrate or product (see Scheme 2). The additional pathways in this mechanism may be regarded as induced-fit steps [22], although in the original description of induced fit, it was not envisaged that such steps would operate in parallel with isomerization pathways. At low substrate concentrations the flows through the induced-fit pathways represent a small fraction of the reaction, but their contributions become progressively greater as the concentration of substrate (or product) is increased. The enzyme therefore effectively changes its mechanism. Rose et al. [20] comment that activation by high substrate concentrations is not a rare phenomenon and their model may have a general significance.

Ricard et al. [23] had previously described how a similar model could produce both positive and negative co-operativity in a monomeric enzyme, and they suggested it to be the explanation of the negative co-operativity that they observed with yeast hexokinase [24]. In their model and considering the hexokinase reaction, the enzyme is released in a form E_{pg} when the products dissociate. At low glucose concentrations, it isomerizes to a form E_{g} before combining with glucose, but, at high glucose concentrations, it combines directly with the sugar. The model is therefore more extreme than that of Rose et al. [20] in that the isomerization step is completely bypassed at high substrate concentrations. A model of this type has also been suggested as the explanation of the sigmoid substrate concentration found with the monomeric enzyme, glucokinase D [25–27].

In the present study, the quantitative consequences on the flux ratios of such additional steps operating in parallel with, and bypassing, enzyme isomerization are examined, using the model of Rose et al. [20] and a model based on that of Ricard et al. [23]. Steady-state velocity equations for the unidirectional fluxes are also derived. The study shows that, in the presence of bypassing steps, there is an additional 1:1 exchange mechanism that gives rise to flux ratios less than expected from the Independence Relationship. To determine whether such additional steps are present, the flux ratios should be studied as a function of substrate and product concentrations. Methods of determining flux ratios from induced-transport curves and analysing the data for the presence of bypassing steps are therefore described. Consideration is also given to the steady-state substrate–velocity curves that may be expected.

To give the studies maximum generality no assumptions have been made about the relative rates of the different steps in the enzyme reactions. Of necessity therefore large numbers of rate constants appear in the equations. For compactness therefore they are denoted by single alphabetical letters. For the same reason, square brackets have not been used to denote concentrations of the substrate and product, although they have been retained for the enzymic species to avoid ambiguities.

FLUX RATIOS WITH A SIMPLE ISOMERIZATION OF THE FREE ENZYME

Considering a single-substrate–single-product reaction ($S \rightleftharpoons P$), the flux ratio may be described by the expression:

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = \frac{SR}{PK} \quad (4)$$

where S and P are the substrate concentrations and K the equilibrium constant as defined in relation to eqn. (1). R represents the factor by which the flux ratio deviates from the Independence Relationship [eqn. (1)].

Scheme 1 represents Scheme 2 with the induced-fit steps omitted. For this scheme, R is given by the expression (see the Appendix):

$$R = \frac{(1 + \sigma KP)}{(1 + \sigma S)} \quad (5)$$

where

$$\sigma = twx(l + g)/[lh(wx + uw + uy)] \quad (6)$$

and t , x , w , etc. are rate constants as indicated in Scheme 1. Substitution of R into eqn. (4) gives essentially the same expression for the flux ratio as previously found [4,8], with σ the equivalent of the term α in the previous work. The additional terms in the expression for σ are the consequence of the additional steps in Scheme 1, in comparison with the minimal homoiokinetic model previously used. When the concentrations of P and S are large enough for σS and σKP to be substantially greater than unity, R approaches KP/S . The flux ratio [eqn. (4)] thus approaches unity and is little affected by the values of S and P . Inverting the equations shows that this will also be the case if the reaction is considered in the reverse direction. Indeed, provided that the equilibrium constant is taken into account, the expression for R [eqn. (5)] is symmetrical with respect to substrate and product. The flux ratio in Scheme 1 approaches unity because the ratio $[E_s]/[E_p]$ (square brackets denote the concentrations of the enzyme species) is reduced below its equilibrium value while the forward reaction is in progress. The relationship is quantitative [4]. Thus if:

$$[E_s]/[E_p] = Zmg/hl \quad (7)$$

where Z is a measure of the displacement of the ratio from its equilibrium value, then $R = Z$ (see the Appendix). The displacement of the ratio depends on the slowness of the isomerization. If the isomerization steps are rapid (g , h , l and $m \rightarrow \infty$), the displacement is small and the flux ratio approximates to that given by the Independence Relationship.

In an approach that is helpful with the more complicated schemes discussed below, let:

$$S = nPK \quad (8)$$

Scheme 1 Isomerization of the free enzyme without bypassing steps

For comparability it is based on Scheme 2 with the omission of the bypassing steps. t , u , x , y , etc. are rate constants. E_s and E_p represent the forms of the enzyme that react with substrate (S) and product (P) respectively.

where n indicates the number of times that the substrate is in excess of its equilibrium value. Thus, from eqns. (4) and (5):

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = nR \quad (9)$$

and

$$R = \frac{(1/P + \sigma K)}{(1/P + n\sigma K)} \quad (10)$$

FLUX RATIOS AND RATE EQUATIONS FOR MECHANISMS IN WHICH ISOMERIZATIONS OF THE FREE ENZYME OPERATE IN PARALLEL WITH STEPS INVOLVING INDUCED FIT

Scheme 2 illustrates the mechanism proposed for fumarase by Rose et al. [20]. The free enzyme exists in three configurations E_s , E and E_p (E_m , $E_{m'}$ and E_r as set out in Scheme 11 of Rose [28]). The substrate and product react with E_s and E_p respectively but the substrate and product may also react with E and 'induce' the isomerization of the enzyme to form the respective binary complexes. For this scheme R is given by the expression (see the Appendix):

$$R = \frac{N + \alpha S + (\beta + \delta)P + (\gamma + \epsilon)SP + \phi P^2}{N + (\alpha + \delta/K)S + \beta P + (\gamma + \phi/K)SP + \epsilon S^2/K} \quad (11)$$

where

$$N = ahm(b + u)(w + q)[(b + u + x)(q + w) + y(b + u)] \quad (12)$$

$$\alpha = abmt(w + q)[(b + u + x)(q + w) + y(b + u)] \quad (13)$$

$$\beta = ahqv(b + u)[(b + u + x)(q + w) + y(b + u)] \quad (14)$$

$$\delta = by[ut(pm + vl)(q + w) + vw(ah + tg)(b + u)] \quad (15)$$

$$\gamma = abtvq[(b + u + x)(q + w) + y(b + u)] \quad (16)$$

$$\epsilon = abtwy(b + u) \quad (17)$$

$$\phi = bptwy(w + q) \quad (18)$$

a , h , m , etc. are rate constants as indicated in Scheme 2. If the rate constants a , b , p and q are zero, the scheme becomes the same as that shown in Scheme 1. The terms N and δ remain finite, but all other terms become zero. Thus σ [eqn. (6)] is equal to $K\delta/N$. If the isomerization steps are rapid, h , l , g and $m \rightarrow \infty$, all of the terms in eqn. (11) disappear apart from N , R is unity and the flux ratio obeys the Independence Relationship (as does Scheme 1 under these conditions). If t , u , v and w are zero, the scheme effectively becomes that of an enzyme with a single form of the free enzyme (E) and R again becomes unity, as is to be expected, since there is no mechanism for the exchange of energy between substrate and product molecules. In the general case, however, R

Scheme 2 A scheme proposed by Rose et al. [20], with bypassing steps that involve reaction of the substrate and product with an intermediate form of the enzyme

t , u , x , y , etc. are rate constants. E_s and E_p react with S and P respectively whereas the enzyme species represented by E can react with either S or P .

is less than unity for reasons that do not simply involve (compare with Scheme 1) the displacement of the ratio $[E_s]/[E_p]$.

Dividing both the numerator and denominator of eqn. (11) by SP and introducing n [eqn. (8)]:

$$R = \frac{N/SP + (n\alpha K + \beta + \delta)/S + \gamma + \epsilon + \phi/nK}{N/SP + (n\alpha K + \beta + n\delta)/S + \gamma + n\epsilon + \phi/K} \quad (19)$$

Inspection of eqn. (19) indicates that at chemical equilibrium ($n = 1$), R is unity for all concentrations of substrate and product, as must be the case. R also remains close to unity when the concentrations of substrate and product are small, since R is then largely determined by N . At intermediate concentrations of substrate and product, the terms $(n\alpha K + \beta + \delta)/S$ and $(n\alpha K + \beta + n\delta)/S$ in the numerator and denominator respectively become important. Restricting the discussion to these terms, if the rate constants a , b , p and q are zero, all terms disappear apart from δ , as already noted. If the rate constants are finite, terms α and β are present. The value of R is increased towards unity by these terms and R becomes dependent on the value of n , even if the N/SP term [eqn. (19)] is small enough to be ignored. For small values of n , R is close to unity. At high values of n , R falls to a limiting value of $1/(1 + \delta/\alpha K)$, and the flux ratio is a constant fraction of that given by the Independence Relationship [eqn. (4)]. In theory, the ratio $\delta/\alpha K$ should be measurable. From eqns. (13) and (15), $\delta/\alpha K \leq (1 + g/l)x/b$, with the equality applying if ES and EP are in rapid equilibrium with E_s and E_p respectively (Scheme 2). Thus it may be possible to make a minimum estimate of the ratio $(1 + g/l)x/b$. If the reaction is considered in the opposite direction, n approaches zero under limiting conditions and the flux ratio becomes a fraction $1/(1 + \delta/\beta)$ of that given by the Independence Relationship. The system is therefore asymmetric in the sense discussed in relation to Scheme 1. From eqns. (14) and (15), $\delta/\beta \leq (1 + l/g)y/q$ with the equality applying if ES and EP are in rapid equilibrium with E_s and E_p . In practice, it may be difficult to determine the ratios $\delta/\alpha K$ and δ/β because, at the high substrate concentrations (or product concentrations if the reverse reaction is considered) required to give sufficiently high values of n (or low values in the case of the reverse reaction), the γ , ϵ and ϕ terms may become important.

At high substrate and product concentrations, the γ , ϵ and ϕ terms may be expected to dominate in eqn. (11). These terms depend entirely on the existence of steps bypassing isomerization of the enzyme and disappear if the rate constants a , b , p and q are zero; further they contain none of the rate constants involved in the isomerization of the enzyme. As at intermediate concentrations, R is dependent on n even if the N/SP term [eqn. (19)] is small. R falls from unity at small values of n to $R = (\gamma + \epsilon)/n\epsilon$ at high values, and from eqn. (4):

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = 1 + \frac{\gamma}{\epsilon} \quad (20)$$

The flux ratio therefore falls to a constant value, at high values of n . If this were demonstrated it would be important evidence for Scheme 2. To determine γ/ϵ [eqn. (20)] from measurements of the flux ratio, $(n-1)/(nR-1)$ may be plotted against n . At sufficiently high concentrations of substrate and product, a straight line should be obtained that has a slope of ϵ/γ and an intercept when $n = 0$ of $(1 + \phi/K\gamma)$.

If the reaction is considered in the reverse direction $n \rightarrow 0$ and:

$$\frac{\text{Flux of P to S}}{\text{Flux of S to P}} = 1 + \frac{K\gamma}{\phi} \quad (21)$$

Scheme 3 Scheme 2 with the rate constants for isomerizations of the free enzyme set to zero

The system is therefore asymmetric in the sense discussed in relation to Scheme 1.

The terms γ/ϵ and $K\gamma/\phi$ [eqns. (20) and (21)] are related to the rate constants as follows:

$$\frac{\gamma}{\epsilon} = \frac{q}{y} \left[1 + \frac{q}{w} + \frac{x(q+w)}{w(b+u)} + \frac{y}{w} \right] \quad (22)$$

and

$$\frac{K\gamma}{\phi} = \frac{b}{x} \left[1 + \frac{b}{u} + \frac{y(b+u)}{u(q+w)} + \frac{x}{u} \right] \quad (23)$$

If ES and E_s and EP and E_p are in rapid exchange ($t, u, v, w \rightarrow \infty$) the terms in square brackets [eqns. (22) and (23)] are unity and the ratios of the coefficients give values for q/y and b/x . In the general case, the terms in square brackets will be greater than unity and thus eqns. (22) and (23) set maximal values for q/y and b/x . Correspondingly, for given values of q/y and b/x the deviation of the flux ratio from the Independence Relationship is greatest when there is rapid exchange, as is also the case at intermediate concentrations (see above).

Scheme 3 represents an extreme variant of Scheme 2, in which E does not isomerize directly to E_s and E_p . The latter enzyme conformations therefore arise only as a result of dissociation of the substrate or product from their respective binary complexes. Under these conditions, N , α , β and δ become zero [eqns. (12)–(15)] and:

$$R = \frac{(\gamma + \epsilon)SP + \phi P^2}{(\gamma + \phi/K)SP + \epsilon S^2/K} \quad (24)$$

Dividing by SP and substituting for n [eqn. (8)]:

$$R = \frac{\gamma + \epsilon + \phi/nK}{\gamma + \phi/K + n\epsilon} \quad (25)$$

These equations are those that apply to the general scheme (Scheme 2) at high substrate and product concentrations. They show that the flux ratio may be less than that expected from the Independence Relationship, although there is no isomerization of the free enzyme in the reaction pathway.

In Scheme 1, the flux ratio is quantitatively less than expected from the Independence Relationship because the net flux from E_p to E_s leads to E_s and E_p departing from their equilibrium proportions by a factor Z [eqn. (7)]. In Scheme 3, there is no direct pathway between E_p and E_s , and, in Scheme 2, at high concentrations of substrate and product, the rate constants for isomerization are not involved in determining the value of R . For Schemes 2 and 3, Z is given by an expression similar to that for R [eqns. (11) and (24)], but in which the coefficients are differently related to the rate constants (see the Appendix). R and Z move in the same direction, when a reaction is in progress, but there is no direct quantitative relationship between them.

Both Schemes 2 and 3 permit a 1:1 exchange between substrate and product molecules, as the result of S and P reversibly dissociating to give E_s and E_p . If, in Scheme 3, the rate constants t , v , u and w are zero, the flux ratio obeys the Independence Relationship (see above). If then it is supposed that the rate constants, t , v , u , w , become finite so that 1:1 exchange can occur, an equal flux is added to the forward and reverse fluxes. The flux ratio is reduced to a value below that required by the Independence Relationship, and the reduction is most marked for large values of n , when the Independence Relationship requires a large difference between the forward and reverse fluxes. In Scheme 3, this represents the only mechanism giving rise to a flux ratio less than expected from the Independence Relationship, and it accounts for the sensitivity of the flux ratio to n , even at high substrate and product concentrations. Similarly, in Scheme 2, it appears to be the only significant mechanism at high substrate and product concentrations. At intermediate concentrations, the δ term may be important representing a contribution from the displacement of the ratio $[E_s]/[E_p]$ from its equilibrium value.

In Scheme 2, despite the existence of a mechanism for 1:1 exchange, if the isomerization steps are rapid and E_s and E_p are in equilibrium, the flux ratio obeys the Independence Relationship.

STEADY-STATE FLUXES AND NET VELOCITIES FOR SCHEMES 2 AND 3

The general expression for the net rate in the steady state is (see the Appendix):

$$V = \frac{[E_t]x[N + \alpha S + (\beta + \delta)P + (\gamma + \epsilon)SP + \phi P^2]S}{bQ(H + AS + BP + CSP + DS^2 + EP^2 + FS^2P + GSP^2)} - \frac{[E_t]xK[N + \beta P + (\alpha + \delta/K)S + (\gamma + \phi/K)SP + \epsilon S^2/K]P}{bQ(H + AS + BP + CSP + DS^2 + EP^2 + FS^2P + GSP^2)} \quad (26)$$

where V is the net velocity, and the terms in large brackets refer to the unidirectional fluxes of S to P and P to S. $[E_t]$ is the total amount of enzyme, and the coefficients α , β , δ , γ , ϵ and ϕ are given by eqns. (12)–(18). Q is defined by eqn. (A19) of the Appendix, and, H , A , B , C , D , E , F and G are also defined in relation to Scheme 2 in the Appendix.

In the absence of product, there is only a flux of S to P. If $a = t(1 + l/m)$, the term in brackets in the numerator of the expression for this flux is a factor of the denominator, and cancellation of this term yields Michaelis–Menten kinetics. If $a < t(1 + l/m)$, the reciprocal plots of velocity against substrate concentration will be concave downwards (negative co-operativity), whereas if $a > t(1 + l/m)$ the reciprocal plots will be concave upwards (positive co-operativity). In the latter case, if the rate constants h and g are sufficiently small, the substrate–velocity curve will inflect upwards and become sigmoid (H. G. Britton, unpublished work). The conditions for sigmoidal curves will be discussed in more detail in relation to Scheme 4. Similar considerations apply to the reverse reaction. However, if both substrate and product are present, cancellation as described above to give Michaelis–Menten kinetics is not possible (except at chemical equilibrium, see below) (H. G. Britton, unpublished work).

In Scheme 3, the rate constants, h , g , l and m in Scheme 2 are zero. Under these conditions, the coefficients N , α , β , δ and H , A and B are also zero, and the net steady-state velocity becomes:

$$V = \frac{E_t x \gamma (S - KP) SP}{bQ(CSP + DS^2 + FS^2P + GSP^2)} \quad (27)$$

From eqn. (27), the velocity is zero in the absence of product as a consequence of the enzyme becoming sequestered in the E_p form. In these circumstances, activation by product should be seen. This may not be a useful property in an enzyme, and it may be unlikely that the rate constants for isomerization will be completely zero as in Scheme 3. Nevertheless, the isomerization steps must be relatively slow for countertransport to be observed and for the enzyme to show either positive or negative co-operativity. At low (physiological) concentrations of substrate the rates of these steps may be adequate to prevent substantial accumulation of E_p and consequent loss of enzyme activity. However, at the much greater turnover rates achieved at high (unphysiological) substrate concentrations, there may be substantial accumulation of E_p , leading to product activation.

If V_e is the flux at chemical equilibrium (equilibrium reaction rate), then (see the Appendix):

$$V_e = \frac{[E_t]x(w + q)(gt/h + a)S}{Q[1 + g/h + l/m + (a/b + p/Kq)S]} \quad (28)$$

The flux at equilibrium therefore shows neither positive nor negative co-operativity [4,20].

REACTION OF SUBSTRATE WITH E_p AND PRODUCT WITH E_s (SCHEME 4)

Scheme 4 is a generalized model, based on that of Ricard et al. [23], that considers the extreme possibility that the substrate may react with E_p and the product with E_s . It differs from the model of Ricard et al. [23] in its provision for a reaction of P with E_s . For Scheme 4 (see the Appendix), R is given by the expression:

$$R = \frac{N + (\alpha + \Delta/K)S + (\beta + \delta)P}{N + (\alpha + \delta/K)S + (\beta + \Delta)P} \quad (29)$$

or from eqn. (8):

$$R = \frac{N/P + (\alpha + \Delta/K)nK + \beta + \delta}{N/P + (\alpha + \delta/K)nK + \beta + \Delta} \quad (30)$$

where

$$N = ae(b + u)(w + q)[(b + u)(w + q + y) + x(w + q)] \quad (31)$$

$$\alpha = abt[(w + q)(w + q + y)(b + u) + x(w^2 + q^2)] \quad (32)$$

$$\beta = apw[(b + u)(w + q)(b + u + x) + y(b^2 + u^2)] \quad (33)$$

$$\Delta = 2abpwuy \quad (34)$$

$$\delta = by(abpq + tuvw) \quad (35)$$

a , b , e , etc. are rate constants as shown in Scheme 4.

At chemical equilibrium $n = 1$ and R [eqn. (30)] is unity, as must be the case. If e and $f \rightarrow \infty$, N is the only coefficient that remains in eqns. (29) and (30) and R is unity under all conditions. This is to be expected since there is effectively only one form of the free enzyme. If a , b , p and q are zero, the mechanism becomes that of Scheme 1 with a single isomerization step and all terms in the expression for R are zero, apart from N and δ . As with Scheme 3, if a , b , p and q are not zero, the additional terms that appear (α , β and Δ) increase the value of R towards unity, and

Scheme 4 A scheme based on a mechanism proposed by Ricard et al. [23]

t, u, x, y , etc. are rate constants. The substrate reacts with E_S , but also with E_P . Correspondingly, the product can react with E_S in addition to E_P . The designation of the two forms of the free enzyme as E_S and E_P is arbitrary. Rotation of E_S and E_P through 180° about an axis in the plane of the paper passing at right angles through the mid-point of the lines joining E_S and E_P causes E_S to be replaced by E_P and vice versa. The rate constants t and u are interchanged with p and q , as are the rate constants v and w and a and b and the rate constants e and f .

make R dependent on n , even when the term N/P [eqn. (30)] is negligible.

In the general case, at high values of P and n [eqn. (30)], R becomes:

$$R = \frac{K\alpha + \Delta}{K\alpha + \delta} \quad (36)$$

The value of R under these conditions thus depends on the relative values of Δ and δ .

From eqns. (34) and (35):

$$\frac{\delta}{\Delta} = \frac{1}{2} \left[\frac{bq}{uw} + \frac{vt}{ap} \right] \quad (37)$$

Also, from eqns. (A25) and (A26) of the Appendix:

$$\frac{bq}{uw} = \frac{ap}{vt} \quad (38)$$

If $X = bq/uw$, then from eqns. (37) and (38):

$$\frac{\delta}{\Delta} = \frac{1}{2} \left[X + \frac{1}{X} \right] \quad (39)$$

The term in square brackets [eqn. (39)] has a minimum value of 2, corresponding to $X = 1$. Under these circumstances, $\Delta = \delta$ and R [eqn. (28)] is unity for all values of n . If X has any other value, δ is greater than Δ and R becomes less than unity. There are no conditions therefore under which the flux ratio can exceed the value given by the Independence Relationship. This is significant in that, in Scheme 4, the reaction pathway taken by a molecule of substrate may be markedly influenced (depending on the rate constants) by the pathway taken by immediately preceding molecules of substrate or product. From eqn. (39), however, this effect does not lead to the transfer of free energy in the way necessary to facilitate the forward reaction and yield a flux ratio greater than that given by the Independence Relationship [see the Introduction and eqn. (3)].

When X is unity, R is unity, and the flux ratio obeys the Independence Relationship under all conditions. However, the conditions for R to be unity [eqn. (29)] are highly unusual. Thus, from eqn. (38), the product of the rate constants for S binding to E_P and P binding to E_S (ap) must be the same as the product of the rate constants for S binding to E_S and P binding to E_P (vt). If the isomerization of E_P to E_S should increase the rate of binding of S, then, for R to be unity, the isomerization E_S to E_P must correspondingly decrease the rate of binding of P to the

enzyme. Rearrangement of eqn. (38) also indicates that, as a general condition, the product of the affinity constant for S binding to E_P and the affinity constant for P binding to E_S must be the same as the product of the affinity constant for S binding to E_S and the affinity constant for P binding to E_P . It would appear that, for R to be unity, the isomerization $E_S \rightleftharpoons E_P$ must, effectively, have no influence on the binding and dissociation of the reactants. In contrast, if the isomerization should favour either binding or substrate or product, as was the intention of the model upon which Scheme 4 is based [22], then $vt > ap$ and $uw > bq$, and, if the inequalities are large, $\Delta \rightarrow 0$. In this context, the designation of the two forms of the free enzyme as E_S and E_P is arbitrary (see the legend to Scheme 4).

If $X \neq 1$, R is less than unity, and, when n and P are large, R falls to a minimum that is independent of n [eqn. (36)]. The flux ratio therefore becomes a constant fraction of that given by the Independence Relationship. This behaviour of the flux ratio differs from that found for Scheme 2 at high substrate and product concentrations, although similar to Scheme 2 at intermediate concentrations.

If it is assumed that the dissociation steps to give E_S and E_P are rapid ($u, w, t, v \rightarrow \infty$), then, from eqn. (39), Δ is zero, and, from eqns. (32), (35) and (36), at high values of P :

$$\frac{1}{R} = 1 + \frac{\delta}{Ka} = 1 + \frac{x}{b} \quad (40)$$

Consideration of eqns. (32), (34) and (35) indicates that the value for x/b given by eqn. (40) is a minimum value, if the assumption of rapid dissociations is not valid.

Correspondingly, if the reaction is considered in the reverse direction, for rapid dissociations:

$$\frac{1}{R} = 1 + \frac{\delta}{\beta} = 1 + \frac{y}{q} \quad (41)$$

As with eqn. (40), the value for y/q will be a minimal value if the assumption that the dissociations are rapid is not correct. Eqns. (40) and (41) indicate that the system is also asymmetrical in the sense discussed in relation to Scheme 2.

From eqns. (22), (23), (40) and (41), the relationships that can be deduced about the rate constants from measurements of the flux ratios at high substrate and product concentrations are very similar with both Schemes 2 and 4.

In addition, Δ is zero if the rate constants a, b, p and q are very rapid. However, this effectively corresponds to reversing the roles and terminology of E_S and E_P (see above and legend to Scheme 4).

Z [defined as $e[E_S]/f[E_P]$, see eqn. (7)] is given by an expression similar to eqn. (29) but the coefficients are related differently to the rate constants [see eqn. (A28)]. As with Scheme 2, if the isomerization is rapid ($e, f \rightarrow \infty$), R is unity and Z is also unity. If $uw = bq$ (see above), R is again unity and, from eqn. (A28), Z is also unity. As discussed above, the isomerization of the enzyme under these last conditions does not on balance favour combination with either the substrate or product, and there is no net flow through the isomerization step while a reaction is taking place. If $uw \neq bq$ (see above), R and Z move in the same direction but are not quantitatively related. For reasons already discussed in relation to Scheme 2, the 1:1 exchange mechanism may play an important role in determining the flux ratio, under these conditions, particularly at high substrate and product concentrations.

If $e, f \rightarrow 0$, the mechanism has similarities to Scheme 3, and the coefficient N [eqn. (29)] becomes zero. R will usually, but not

necessarily (see above), be less than unity. In contrast, if $e, f \rightarrow \infty$, R is always unity as has been discussed.

STEADY-STATE FLUXES AND NET VELOCITIES FOR SCHEME 4

For Scheme 4 the steady-state net velocity V is given by (see the Appendix):

$$V = \left[\frac{[E_t]x[N+(\alpha+\Delta/K)S+(\beta+\delta)P]S}{bL(H+AS+BP+CSP+DS^2+EP^2)} \right] - \left[\frac{[E_t]xK[N+(\alpha+\delta/K)S+(\beta+\Delta)P]P}{bL(H+AS+BP+CSP+DS^2+EP^2)} \right] \quad (42)$$

The terms in large brackets refer to the unidirectional fluxes of S to P and P to S respectively. $[E_t]$ is the total amount of enzyme, L is defined by eqn. (A31) and H, A, B, C, D and E are also defined in the Appendix in relation to Scheme 4.

If $t = a$, in the absence of product, Michaelis–Menten kinetics are obeyed, since the term in brackets in the numerator of eqn. (42) for the flux of S to P is a factor of the denominator, and similarly, if $v = p$, for the reverse reaction. In addition, however, if $uw = bq$, then the terms in square brackets in the numerators for the flux of S to P and the flux of P to S are the same, and a factor of the denominator. Cancellation of this factor yields the equation to be expected for an enzyme with a single form of the free enzyme. As already discussed, and in agreement with this conclusion, the flux ratio obeys the Independence Relationship under these conditions. Thus the isomerizations shown in Scheme 4 cannot be detected by steady-state kinetics. The requirement that $bq = uw$ is met if $t = a$ and $v = p$, and also by the less stringent condition that $ap = vt$ [eqn. (38)].

If $t \neq a$ and $bq \neq uw$, Scheme 4 cannot give Michaelis–Menten kinetics. If the product $(t-a)(uw-bq)$ is positive, reciprocal plots of initial velocity against substrate concentration will be concave downwards, corresponding to negative co-operativity, whereas if $(t-a)(uw-bq)$ is less than zero, the plots will be concave upwards corresponding to positive co-operativity. Similar conclusions were reached by Ricard et al. [23] for their simpler model, which can be derived from Scheme 4 by setting the rate constants p and q to zero. That $(t-a)(uw-bq)$ should be less than zero is also the necessary condition, provided that the rate constants e and f are sufficiently small for the substrate–velocity curves to inflect upwards to give sigmoidal plots (H. G. Britton, unpublished work).

If $bq \neq uw$, R [eqn. (29)] is potentially less than unity, and the flux ratio is less than given by the Independence Relationship, whether positive or negative co-operativity is shown. To consider the flux ratios under conditions under which sigmoidal substrate–velocity curves are obtained, the generation of such curves requires quite extreme conditions (H. G. Britton, unpublished work). Thus the equilibrium between E_s and E_p must be highly asymmetric. Further, if, for example, the equilibrium lies towards E_s (Scheme 4) the major pathway for the enzyme reaction must involve the intermediates, E_p , ES and EP . Model building suggests that, at the steepest part of the Hill plot, a model giving a sigmoid plot should yield a value of R appreciably less than unity, and there should be a further fall in R at higher substrate concentrations. However, the system will also be markedly asymmetrical with respect to the effects of substrate and product on the value of R , and, unless the concentration of the product is small, R may be close to unity.

The ability of Scheme 4 to generate sigmoidal substrate–velocity curves is not affected if the rate constants p and q are set to zero (H. G. Britton, unpublished work). Using the equivalent

reaction approach (see the Appendix), Scheme 2, in the absence of products, can be shown to be effectively the same as Scheme 4 without the steps represented by the rate constants p and q . It follows that Scheme 2 can generate substrate–velocity curves of a similar degree of sigmoidicity to those generated by Scheme 4.

If V_E represents the flux at chemical equilibrium (equilibrium reaction rate) then (see the Appendix):

$$V_E = \frac{[E_t]x(w+q)(ae/f+t)S}{L[1+e/f+(t/u+p/Kq)S]} \quad (43)$$

As with Scheme 2, the equilibrium reaction rate with Scheme 4 does not show either positive or negative co-operativity [4,20].

A GENERAL TEST FOR THE PRESENCE OF STEPS BYPASSING ENZYME ISOMERIZATION

If there is a simple isomerization of the free enzyme, the flux ratio is determined by one constant σ [eqn. (5)], and this factor may be calculated from a single measurement of the flux ratio. Thus, to determine whether bypassing steps are present, it may be sufficient to determine σ for a wide range of conditions, preferably involving the reverse as well as the forward reaction. If the value of σ is not constant and tends to fall as equilibrium is approached, it will be evidence for the presence of bypassing steps. A more systematic approach may be as follows. Let

$$F_r = \frac{\text{Flux of } S \text{ to } P}{\text{Flux of } P \text{ to } S} \quad (44)$$

Then, since

$$\text{Net flux of } S \text{ to } P = [\text{Flux of } S \text{ to } P] - [\text{Flux of } P \text{ to } S]:$$

$$\frac{\text{Flux of } S \text{ to } P}{\text{Net Flux of } S \text{ to } P} = \frac{F_r}{F_r - 1} \quad (45)$$

For Scheme 2, from this relationship, and eqns. (4) and (11):

$$\frac{F_r(S-KP)}{(F_r-1)S} = 1 + \frac{P\delta}{N} \left[\frac{(1+\phi P/\delta + \epsilon S/\delta)}{(1+\beta P/N + \alpha S/N + \gamma SP/N)} \right] \quad (46)$$

If the rate constants for the steps bypassing the enzyme isomerization are zero ($a, b, p, q \rightarrow 0$), the term in large brackets on the right hand side of eqn. (46) has a value of unity. However, if the rate constants for the steps are finite, then, from eqns. (12)–(15), (17), (18), (A4) and (A5), $\phi/\delta < \beta/N$ and $\epsilon/\delta < \alpha/N$. The term in large brackets therefore has a value of less than unity and its value progressively decreases as the concentrations of S and P are increased.

For Scheme 4:

$$\frac{F_r(S-KP)}{(F_r-1)S} = 1 + \frac{(\delta-\Delta)P}{N} \left[\frac{1}{(1+\alpha S/N + \beta P/N)} \right] \quad (47)$$

The term in large brackets on the right hand side of eqn. (47) again has a value of unity, if the rate constants bypassing the enzyme isomerization are zero ($a, b, p, q \rightarrow 0$), and its value becomes less than unity if the rate constants are finite. Thus, flux ratios should be measured at a series of concentrations of substrate and product, and with the net reaction taking place in both directions. These data should be plotted in the form given by the left-hand sides of eqns. (46) and (47), against the concentration of P . If there is a simple isomerization of the enzyme and the rate constants for the bypassing steps are zero ($a, b, p, q \rightarrow 0$), all of the data should fall on a single straight line that should pass through unity at zero concentration of P , and should have a slope that is independent of the concentration of S . In contrast, if the rate constants for the bypassing steps are

experiment in which the data for the substrate have been plotted as a function of the concentration of the substrate (S) (left hand panel) and the data for the product have been plotted as a function of the concentration of product (P) (right hand panel). Initially there is equilibrium and the concentrations of substrate and product are S_o and P_o . P_o is higher than S_o , in the example shown, because the equilibrium constant [defined in relation to eqn. (1)] is less than unity (0.25). For the same reason, the amount of label per unit volume in the product (N_p) is greater than in the substrate (N_s). At zero time, unlabelled substrate is added to give a total concentration of S_x , and, while the concentration of S falls to its final equilibrium value S_e , since countertransport is illustrated, a transient rise in N_s is shown corresponding to a flow of labelled material in the opposite direction to the net flow. There is a corresponding and equal fall in the amount of label in the product.

The maximum amount of countertransport occurs when the flux ratio is very close to unity and the exchange of label between the reactants is rapid compared with the net reaction. When unlabelled S is added, under these conditions, the specific radioactivities of the substrate and product become identical from the outset. N_s rises to the point marked 'Max N_s ' (Figure 1), and subsequently the amount of label in S follows a straight line, which reflects the fall in concentration of S and which passes through the point N_s at chemical equilibrium (Figure 1, left hand panel). Correspondingly, the amount of label in the product falls rapidly to a minimum with a linear return as the reaction proceeds and product accumulates (Figure 1, right hand panel). When these limits to the maximum amount of countertransport are approached, as may happen in the later stages of the reaction, the induced transport curve largely reflects the subsequent change in concentration of the substrate and is insensitive to the flux ratio.

Apart from the extreme case when the substrate and product have come into isotopic equilibrium, the instantaneous slope of the induced transport curve is related to the flux ratio. If, at point X (Figure 1), the properties of the enzyme should change so that the flux ratio obeys the Independence Relationship, the curve becomes a straight line passing through the point of final equilibrium (dashed lines in Figure 1) [8]. If, at point X, the properties of the enzyme should change to give a flux ratio greater than that given by the Independence Relationship, the line would have a steeper (more negative) slope. However, none of the mechanisms considered in the present study can yield such a flux ratio and therefore the induced-transport curves generated by the models cannot approach the horizontal, asymptotically. Thus the area under the induced transport curve, which might be used as a measure of the amount of countertransport, is likely to be largely a reflection of the flux ratios occurring during the initial part of the reaction, particularly if the amount of countertransport is substantial.

To calculate the flux ratios from the instantaneous slope of the induced-transport curve, eqn. (4) may be rearranged as follows:

$$\frac{\text{Flux of S to P}}{\text{Net flux of S to P}} = \frac{SR}{SR - PK} \quad (48)$$

and

$$\frac{\text{Flux of P to S}}{\text{Net Flux of S to P}} = \frac{PK}{SR - PK} \quad (49)$$

At any instant in time, the amount of label in the substrate will be $(N_s + \Delta N_s)$, where ΔN_s represents the amount of countertransport. Correspondingly, the amount of label in the product

will be $(N_s/K - \Delta N_s)$ (Figure 1). The specific radioactivities of S and N may be obtained by dividing the amounts of label by the concentrations. Thus, as previously described [8,10], from eqns. (48) and (49):

$$M_s = \frac{dN_s}{dS} = \frac{R(N_s + \Delta N_s) - K(N_s - \Delta N_s/K)}{SR - PK} \quad (50)$$

where M_s is the slope of the induced-transport curve. Rearranging eqn. (50)

$$R = \frac{M_s PK - N_s + \Delta N_s K}{M_s S - N_s - \Delta N_s} \quad (51)$$

Since the concentration of S falls throughout the reaction, the slope, M_s , is negative, while the amount of label in S is increasing. With large rates of increase, $M_s \rightarrow -\infty$, and, from eqns. (51) and (8), $R \rightarrow 1/n$, corresponding to a flux ratio of unity. The accuracy of the procedure is likely to be at its greatest during the early stages of the reaction, particularly if considerable exchange occurs. If the dependence of R on the concentrations of S and P during the reaction can be established for much of the reaction, it may be useful to integrate the relationship numerically [10] to check the overall fit of the derived data to the induced-transport curve. If the flux ratio obeys the Independence Relationship ($R = 1$) and using the substitution $(S - PK) = (S - S_e)(1 + K)$, eqn. (50) may be used to verify that the straight line shown in Figure 1 passes through the point of final equilibrium.

Eqn. (51) also applies to induced-transport tests carried out under non-classical conditions. The appropriate straight line for N_s is drawn corresponding to the flux ratio obeying the Independence Relationship, and ΔN_s is the difference between the measured radioactivity and the straight line (see the dashed line in Figure 1 [8]). If all of the label is initially in the product, small changes in the amount of label in the substrate may be more readily detected in the early stages of the reaction. However, in such experiments the substrate concentration during the reaction must be known accurately. The classical induced-transport test is more tolerant in this respect, particularly if the major objective is to determine the direction of the deviation from the Independence Relationship.

DISCUSSION

If there is a simple isomerization of the enzyme (Scheme 1), the deviation of the flux ratio from the Independence Relationship is quantitatively related to the reduction of the ratio $[E_s]/[E_p]$ below its equilibrium value, as a consequence of the net flow of E_p to E_s , when the reaction is in progress. Under these circumstances the deviation from the Independence Relationship is specified by a single constant $[\sigma]$, eqn. (5) in whichever direction the reaction is studied. Further, at high substrate and product concentrations, the flux ratio remains close to unity, for all values of substrate and product concentration. The present study shows, however, that, when bypassing steps are present, additional terms appear in the flux ratio equations that have the effect of reducing the deviation of the flux ratio from the Independence Relationship. Nevertheless, when the reaction is far from equilibrium $\{n \gg 1$ [eqn. (8)]\}, the deviation from the Relationship can be very large, even though the ratio approximates to the Independence Relationship as equilibrium is approached. This is the case both with Scheme 2, in which the isomerization of the enzyme is partially bypassed, and Scheme 4, in which the isomerization of the enzyme is completely bypassed.

In the presence of bypassing steps, both Scheme 2 and Scheme 4 behave asymmetrically in that different terms determine the

flux ratios in the two directions. Further, the flux ratios are not simply related to the change in the ratio $[E_s]/[E_p]$, and the rate constants for isomerization are not important in determining the flux ratios at high substrate and product concentrations. Scheme 3, a variant of Scheme 2, in which the rate constants for isomerization are zero, also gives rise to flux ratios less than expected from the Independence Relationship, despite the absence of any flow through the isomerization step. The difference in the behaviour of the flux ratios when bypassing steps are present is the consequence of a 1:1 exchange of substrate and product molecules, as the result of ES dissociating reversibly to E_s and EP dissociating reversibly to E_p . This novel mechanism affects the flux ratio to the greatest extent when the reaction is far from equilibrium. Its contribution to the flux ratio is also greatest at high substrate and product concentrations. Measurement under such conditions may make it possible to distinguish between Schemes 2 and 4, and set minimum values for the rate constants for interconversion of ES and EP, in relation to the rates of dissociation of S and P via their respective bypass pathways.

Despite the fact that, when bypassing steps are present, there is a contribution to the flux ratio from a novel mechanism, all of the schemes give flux ratios that obey the Independence Relationship, if the rate constants for the isomerization of E_s to E_p and vice versa are large and the two forms of the enzyme are in equilibrium. A flux ratio less than that expected from the Independence Relationship and countertransport therefore remains strong evidence for the existence of two forms of the free enzyme, one reacting preferentially with substrate and the other reacting preferentially with product, and that these forms are not in rapid exchange.

In Scheme 4, in which the enzyme isomerization steps may be completely bypassed by the induced-fit steps, under unusual conditions, steady-state kinetics, non-isotopic or isotopic, may give no indication of the presence of enzyme isomerization. However, the conditions require that the isomerization $E_s \rightleftharpoons E_p$ is effectively neutral in its effects on binding of the substrate and product. Under no conditions does the mechanism lead to a flux ratio in excess of that given by the Independence Relationship. This is despite the fact that when one molecule of substrate reacts with the enzyme it may greatly influence the reaction pathway of the next molecule of substrate. With a suitable choice of rate constants, for example, consecutive turnovers of the enzyme might involve E_s and E_p alternately. Such an interaction between successive molecules of substrate reacting with the enzyme might have conceivably given a flux ratio greater than expected from the Independence Relationship.

Countertransport remains very strong evidence for the existence of two forms of the enzyme (E_s and E_p) that are not in rapid exchange, since it demonstrates that the flux ratio is less than that expected from the Independence Relationship. However, if the reaction is to be examined for the presence of bypassing steps, flux ratios must be determined. The flux ratios may be measured directly, or, as is described, they may be derived from induced-transport curves. The induced-transport test and the direct measurement of flux ratios are closely related, and the derivation of flux ratios from induced-transport curves may be the most efficient way of utilizing the experimental data.

From the flux ratios, it may be self-evident that bypassing steps are present. Thus, assuming a simple isomerization, σ [eqn. (5)] may be calculated from the flux ratios derived from the early stages of the reaction. If the flux ratios calculated from the data become greater than expected from eqn. (5), as equilibrium is approached, it will be strong evidence for the presence of an induced-fit step bypassing the isomerization of the enzyme. The

reaction should also be studied in the reverse direction, since a value of σ differing from that calculated for the forward reaction will be strong evidence for the presence of bypassing steps. In less clear-cut cases, the data may be plotted as described in relation to eqns. (46) and (47).

In the presence of bypassing steps, both Schemes 2 and 4 may show either positive or negative co-operativity [23,27]. In both cases the flux ratio should be less than expected from the Independence Relationship. With both schemes, the positive co-operativity may be sufficiently extreme for sigmoid substrate-velocity curves to be obtained. As has been discussed, the conditions necessary to give sigmoid curves are such that it may be difficult to observe deviations from the Independence Relationship because of the asymmetry of the system (H. G. Britton, unpublished work). A unique feature of Scheme 2 is the possibility that, at high substrate concentrations, product activation may be seen.

With both Schemes 2 and 4, the flux at chemical equilibrium (equilibrium reaction rate) should not show either positive or negative co-operativity, since equilibrium reaction rates are insensitive to isomerization steps [4,21]. It would seem important to establish a lack of co-operativity in the equilibrium reaction rate if positive or negative co-operativity is to be attributed to the presence of steps bypassing an isomerization of the enzyme.

The presence of induced-fit steps in parallel with isomerizing steps implies that, during the process of induced fit, the substrate retains its ability to dissociate and exchange with the substrate in solution, whereas an increase in affinity of binding associated with induced fit might be expected to decrease the rate of dissociation. However, the activator for rat carbamoylphosphate synthetase, acetylglutamate, has been shown to remain in rapid exchange with the solution despite a slow activation process taking many minutes that is associated with a very marked increase in affinity of the enzyme for acetylglutamate [31,32].

Binding of substrates must, almost certainly, be a multistep process and may be chaotic. In the latter case, it may not be possible to define, in exact molecular detail, any particular binding pathway. Further, Scheme 4 may represent an extreme of a spectrum of binding, with Scheme 2 an intermediate in the spectrum. The binding process may thus be a continuum, with the substrate becoming more able to bind as the enzyme progressively assumes the configuration represented by E_s . Such a mechanism is perhaps likely to be approximated by Scheme 2, although much depends on the rate constants and Scheme 4 may be a better approximation in some cases. Agreement with any particular model may therefore not be exact. Nevertheless, the induced-transport/flux ratio technique should permit an insight into events that are not readily accessible using other techniques.

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APPENDIX

Calculation of flux ratios

Scheme 1

The procedure described for Scheme 2 (see below) yields the flux ratio in terms of the ratio $[E_s]/[E_p]$. Thus, using square brackets to denote the concentrations of the enzyme species:

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = \frac{S}{PK} \times \frac{h[E_s]}{gm[E_p]} \quad (\text{A1})$$

The ratio of the enzymic species as functions of the rate constants may be obtained by calculating the fluxes between them (see Scheme 2), or by the King and Altman [1] procedure.

Scheme 2

The free substrate and product in solution may be regarded as located in compartments, and, in the process of conversion from substrate into product, the substrate has to pass through two ‘enzyme-bound compartments’, ES and EP. The overall flux ratio is the product of the flux ratios for the passage from one compartment to another [2]. Thus, following Britton [3]:

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = \frac{(St[E_s] + Sa[E])}{(b+u)[ES]} \times \frac{x[ES]}{y[EP]} \times \frac{(q+w)[EP]}{(Pv[E_p] + Pp[E])} \quad (\text{A2})$$

Further:

$$K = bpy/ax \quad (\text{A3})$$

where K is the equilibrium constant for the reaction (S_{eq}/P_{eq} , S_{eq} and P_{eq} being equilibrium values).

Also:

$$ahu = bgt \quad (\text{A4})$$

and

$$mpw = lvq \quad (\text{A5})$$

From eqns. (A2), (A3) and (4):

$$R = \frac{bp(w+q)(t[E_s] + a[E])}{aq(b+u)(v[E_p] + p[E])} \quad (\text{A6})$$

To obtain a full solution from eqn. (A6), the relative quantities of E_s , E_p and E must be expressed as functions of the rate constants. The fluxes between these enzyme species were therefore calculated, since the fluxes in both directions between any pair must be equal [4]. This procedure yields the relative proportions of the enzyme species rather than the absolute amounts (but see Scheme 4, below), but, involves less computation than the equivalent reaction technique [4]. The most complicated scheme for which a solution is readily available is used as a starting point and the final solution is obtained by introducing the additional species. In introducing the additional species, the rate constants in the simpler scheme are replaced by ‘complex’ rate constants

[e.g. eqns. (9)–(14)]. These complex rate constants, when multiplied by the concentration of the enzyme species, give the appropriate fluxes. Thus, for example, using the substitution in eqn. (A10), the flux of E to E_s in Figure A1, scheme II, is the same as that in Figure A1, scheme I. At each step the result is a ratio, and, if the calculation involves taking products of pairs of complex rate constants, there will be a common factor in the numerator and denominator. If a star-delta transformation is involved ([4], and see below), the factor is $(b+c+d)$, where b , c and d are the rate constants radiating from the enzyme species that is being introduced; if the step is an intermediate in an unbranched pathway, the factor is $(b+c)$, where b and c are the rate constants for the forward and reverse reactions. For the simpler schemes, cancellation is not required, and several additional species can be introduced in the same algebraic step. However, for the more complicated schemes, where cancellation is required, the additional species may be introduced sequentially to avoid mathematical complication. For the present scheme the starting point was Figure A1, scheme I. For this scheme,

$$\frac{[E_s]}{[E]} = \frac{a''d'' + a''e'' + f''d''}{b''d'' + b''e'' + c''e''} \quad (\text{A7})$$

and

$$\frac{[E_p]}{[E]} = \frac{f''b'' + f''c'' + a''c''}{b''d'' + b''e'' + c''e''} \quad (\text{A8})$$

These solutions can be readily derived by the flux ratio method or by other techniques.

To introduce the EP species into Figure A1, scheme I to give scheme II a star-delta transformation was used [4], and, the following substitutions were made into eqns. (A7) and (A8):

$$b'' = h' + \frac{a'y'}{b' + y' + w} \quad (\text{A9})$$

$$a'' = g' + \frac{b'x'}{b' + y' + w} \quad (\text{A10})$$

$$c'' = \frac{a'w}{b' + y' + c'} \quad (\text{A11})$$

$$d'' = \frac{b'v}{b' + y' + w} \quad (\text{A12})$$

$$e'' = m + \frac{vy'}{b' + y' + w} \quad (\text{A13})$$

$$f'' = l + \frac{wx'}{b' + y' + w} \quad (\text{A14})$$

Figure A1 Calculation of the relative concentrations of E_s , E and E_p (Scheme 2 of main paper)

Schemes I and II represent intermediate stages in the calculations. The primes and double primes indicate that the rate constants are derived values. For further details see the text.

Insertion of these equations into eqn. (A7) yields, after cancellation of $(b' + y' + w)$ from the numerator and denominator:

$$\frac{[E_s]}{[E]} = \frac{h'l(b' + y' + w) + a'l(w + y') + wx'(h' + a') + a'wg'}{mh'(b' + y' + w) + vh'(b' + y') + a'm(w + y') + a'y'v} \quad (\text{A15})$$

Substitution into eqn. (A8) yields a similar equation for the ratio $[E_p]/[E]$.

To obtain the corresponding equations for Scheme 2 of the main paper, a star-delta transformation was carried out introducing ES from Scheme 2 in the main paper into scheme II of Figure A1. The complex rate constants so derived were inserted into eqn. (A15) and into the corresponding equation for $[E_p]/[E]$.

Insertion of the ratios obtained above into eqn. (A6) yielded eqn. (11) of the main paper. The expressions for the coefficients had p as a common factor. This rate constant was therefore deleted to yield eqns. (12)–(18) of the main paper.

The numerators and denominators obtained for the ratios of the enzyme species are the same as those obtained by the King and Altman method [1], although only purely algebraic manipulations are involved.

The expression for Z [eqn. (7) of the main paper] is:

$$Z = \frac{N + \alpha KS + (\beta + \delta)P + (\gamma + \epsilon)SP + \phi P^2}{N + (\alpha + \delta/K)S + \beta P + (\gamma + \phi/K)SP + \epsilon S^2/K} \quad (\text{A16})$$

where $N = lmg[(q + w)(b + u + x) + y(b + u)]$, $\alpha = almu(q + w + y)$, $\beta = pmgw(b + u + x)$, $\delta = ly[u(pm + lv) + vg(b + u)]$, $\gamma = alvqu$, $\phi = lpuy$ and $\epsilon = alwv$. When calculated, all of the expressions for the coefficients contained a common factor, h , which was deleted to give those shown.

Calculation of steady-state fluxes for Scheme 2 of the main paper

Following [3], the flux of S to P for Scheme 2 is given by the expression:

$$\text{Flux of } S \text{ to } P = x(w + q)(tE_s + aE)S/Q \quad (\text{A17})$$

and

$$\text{Flux of } P \text{ to } S = y(b + u)(vE_s + pE)P/Q = \quad (\text{A18})$$

where

$$Q = (w + q)(b + u + x) + y(b + u) \quad (\text{A19})$$

The amounts of E_s , E and E_p [eqns. (A17) and (A18)], are related to the total amount of enzyme, E_t , by an expression of the form $[\text{Numerator}]/[\text{Denominator}]$, where the denominator ('King and Altman denominator', see below) is common to all of the expressions [1]. The appropriate numerators are suitably incorporated in the expression for R (eqn. 11) for direct substitution into eqns. (A17) and (A18), provided that the rate constant, p , is replaced in the expressions for the coefficients. Thus, rearranging

Figure A2 Calculation of the King and Altman [1] denominator for Scheme 2 of the main paper, to express E_s , E and E_p in terms of the total enzyme E_t

Schemes I and II represent intermediate stages in the calculations. For further details see the text.

eqn. (A17) and comparing with the expressions for R in eqns. (A6) and (11):

$$\begin{aligned} \text{Flux of } S \text{ to } P &= xS[bp(w + q)(t[E_s] + a[E])]/bpQ \\ &= xS[\text{NUM}][E_s]/(bQ[\text{KDEN}]) \end{aligned} \quad (\text{A20})$$

Similarly, substituting for y in eqn. (A18) using eqn. (A3) and comparing with eqns. (A6) and (11):

$$\begin{aligned} \text{Flux of } P \text{ to } S &= xPK[aq(b + u)(v[E_s] + p[E])]/bpQ \\ &= PK[\text{DEN}][E_s]/(bQ[\text{KDEN}]) \end{aligned} \quad (\text{A21})$$

where $[\text{NUM}]$ and $[\text{DEN}]$ represent the numerator and denominator of eqn. (11), and $[\text{KDEN}]$ represents the 'King and Altman denominator'.

$[\text{KDEN}]$ was calculated using the 'equivalent reaction' technique [4]. For scheme I of Figure A2, the King and Altman [1] denominator, D , is:

$$D = y'(a' + p' + b') + q'(a' + b' + x') + x'(a' + p') + b'p' \quad (\text{A22})$$

The enzyme species E_s was introduced (scheme II of Figure A2), by substitution of the appropriate complex rate constants into eqn. (A22) with cancellation of the common factor $(h + t)$ (see above). The enzyme species E_p was then introduced by appropriate substitutions to give Scheme 2 of the main paper with cancellation of the common factor $(v + m)$. The expression for D thus obtained is the required denominator despite the fact that no direct consideration was given to the amounts of the enzyme species, E_s and E_p . This arises from the compensation that is applied in the equivalent reaction technique to take into account the amounts of E_s and E_p . The steady-state fluxes are given as eqn. (26) of the main paper. The values of the coefficients in this equation are as follows:

$$H = [h(m + l) + gm][by + q(b + x) + u(q + y) + w(b + u + x)]$$

$$\begin{aligned} A &= y[am(h + u) + bt(m + l)] + q[am(h + u) \\ &\quad + t(b + x)(m + l)] + x(ah + gt)(m + w) + gmt(w + q + y) \\ &\quad + tw(b + x)(m + l) + amw(h + u) \end{aligned}$$

$$\begin{aligned} B &= y[(pm + vl)(h + u) + bv(h + g)] + vq(b + x)(h + g) \\ &\quad + ph(m + w)(b + x + u) + uv(q + y)(h + g) + hlv(b + x + u) \end{aligned}$$

$$\begin{aligned} C &= y[av(h + u) + t(pm + vl) + bte] + qv[ah + u \\ &\quad + t(b + x)] + vx(ah + gt) + pt(b + x)(m + w) \\ &\quad + gtv(q + y) + ltv(b + x) \end{aligned}$$

$$D = at[m(y + q + x + w) + wx]$$

$$E = pv[h(u + y + x + b) + uy]$$

$$F = atv(q + x + y)$$

$$G = ptv(b + x + y)$$

Figure A3 Scheme used in the calculation of the relative proportions of E_s , E_p , ES and EP in Scheme 4 of the main paper

The primes indicate that the rate constants are derived values. For further details see the text.

The flux at chemical equilibrium (equilibrium reaction rate) was calculated from eqn. (A17), the quantities of E_s and E as a function of E_t being derived assuming chemical equilibrium.

Calculation of the flux ratios for Scheme 4 of the main paper

Following the argument for Scheme 2, for Scheme 4 of the main paper:

$$\frac{\text{Flux of S to P}}{\text{Flux of P to S}} = \frac{(St[E_s] + Sa[E_p])}{(b+u)[ES]} \times \frac{x[ES]}{y[EP]} \times \frac{(w+q)[EP]}{(Pv[E_p] + Pp[E_s])} \quad (\text{A23})$$

Also,

$$bvy/awx = K \quad (\text{A24})$$

where K is the equilibrium constant of the reaction,

$$aeu = bft \quad (\text{A25})$$

and

$$fpw = evq \quad (\text{A26})$$

From eqn. (A25), and eqn. (4) of the main paper:

$$R = \frac{bv(w+q)(a[E_p] + t[E_s])}{aw(b+u)(v[E_p] + p[E_s])} \quad (\text{A27})$$

To determine the ratio $[E_s]/[E_p]$ (required in eqn. A27), the Scheme in Figure A3 was used as a starting point, and EP was introduced by regarding this scheme as having arisen from Scheme 4 of the main paper by a star-delta transformation with elimination of EP. The final result of these calculations is shown in eqn. (29) of the main paper. The coefficients had the rate constant, v , as a common factor which was deleted in the expressions for the coefficients given in eqns. (31)–(35) of the main paper.

The expression for Z [eqn. (7) of the main paper] is:

$$Z = \frac{N + (\alpha + \Delta/K)S + (\beta + \delta)P}{N + (\alpha + \delta/K)S + (\beta + \Delta)P} \quad (\text{A28})$$

where

$$N = e f [(b+u)(q+w+y) + x(q+w)]$$

$$\alpha = a e u (q+w+y)$$

$$\beta = f p w (b+u+x)$$

$$\Delta = b f p y$$

$$\delta = e v y u$$

Calculation of steady-state fluxes for Scheme 4 of the main paper

For Scheme 4, following Britton [3]:

$$\text{Flux of S to P} = x(w+q)(aE_p + tE_s)S/L \quad (\text{A29})$$

and

$$\text{Flux of P to S} = y(b+u)(vE_p + pE_s)P/L \quad (\text{A30})$$

where

$$L = (w+q)(b+u+x) + y(b+u) \quad (\text{A31})$$

Following the procedure described in relation to Scheme 2, and taking into account the deletion of the rate constant, v , in the expressions for the coefficients in eqn. (29) of the main paper:

$$\text{Flux of S to P} = xS[\text{NUM}][E_t]/(bL[\text{KDEN}]) \quad (\text{A32})$$

and

$$\text{Flux of P to S} = xPK[\text{DEN}][E_t]/(bL[\text{KDEN}]) \quad (\text{A33})$$

The terms in [NUM] and [DEN] represent the numerator and denominator of eqn. (29) of the main paper.

[KDEN] is the King and Altman denominator to express the fluxes in terms of the total amount of enzyme, E_t . [KDEN] was obtained by summing the numerator and denominator of the expression for $[E_s]/[E_p]$ with the numerator of the ratio $[ES]/[E_p]$ and the numerator of the ratio $[EP]/[E_p]$ (obtained by symmetry). This denominator yielded the following values for the coefficients in the denominator of eqn. (42) of the main paper.

$$H = (e+f)[(b+u)(w+q+y) + x(w+q)]$$

$$A = (w+q+y)[a(e+u) + t(b+f)] + x[a(q+e) + t(f+w)]$$

$$B = (b+u+x)[p(f+w) + v(q+e)] + y[p(b+f) + v(e+u)]$$

$$C = vt(b+q+x+y) + ap(w+u+x+y)$$

$$D = at(w+q+x+y)$$

$$E = pv(b+u+x+y)$$

The flux at chemical equilibrium (equilibrium reaction rate) was derived using eqn. (A29), assuming chemical equilibrium to calculate the concentrations of E_s and E_p in terms of E_t .

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