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Generalized Microscopic Reversibility, Kinetic Co-operativity of Enzymes and Evolution

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Generalized microscopic reversibility implies that the apparent rate of any catalytic process in a complex mechanism is paralleled by substrate desorption in such a way that this ratio is held constant within the reaction mechanism [Whitehead (1976) *Biochem. J.* **159**, 449–456]. The physical and evolutionary significances of this concept, for both polymeric and monomeric enzymes, are discussed. For polymeric enzymes, generalized microscopic reversibility of necessity occurs if, within the same reaction sequence, the substrate stabilizes one type of conformation of the active site only. Generalized microscopic reversibility suppresses the kinetic co-operativity of the slow transition model [Ainslie, Shill & Neet (1972) *J. Biol. Chem.* **247**, 7088–7096]. This situation is obtained if the free-energy difference between the corresponding transition states of the two enzyme forms is held constant along the reaction co-ordinate. This situation implies that the 'extra costs' of energy (required to pass each energy barrier) that are not covered by the corresponding binding energies of the transition states vary in a similar way along the two reaction co-ordinates. The regulatory behaviour of monomeric enzymes is discussed in the light of the concept of 'catalytic perfection' proposed by Albery & Knowles [(1976) *Biochemistry* **15**, 5631–5640]. These authors claim that an enzyme will be catalytically 'perfect' when its catalytic efficiency is maximum. If this situation occurs for a monomeric enzyme obeying either the slow transition or the mnemonical model, it can be shown that the kinetic co-operativity disappears. In other words, kinetic co-operativity of a monomeric enzyme is 'paid for' at the expense of catalytic efficiency, and the monomeric enzyme cannot be simultaneously co-operative and catalytically very efficient. This is precisely what has been found experimentally in a number of cases.

Kinetic co-operativity, that is the seemingly co-operative or antico-operative kinetic behaviour of enzymes, is the consequence of subunit interactions and (or) of the occurrence of several closed loops involving the substrate in the reaction mechanism. With this operational definition, a monomeric enzyme can exhibit co-operativity (Rabin, 1967; Frieden, 1970; Ainslie *et al.*, 1972; Ricard *et al.*, 1974*b*; Meunier *et al.*, 1974; Shill & Neet, 1975; Ricard *et al.*, 1977; Buc *et al.*, 1977; Storer & Cornish-Bowden, 1977; Monneuse-Doublet *et al.*, 1978) and a polymeric enzyme must, as a rule, exhibit a kinetic co-operativity that is not adequately expressed by its substrate-binding isotherm (Whitehead, 1970; Wong & Endrenyi, 1971; Endrenyi *et al.*, 1971; Ricard *et al.*, 1974*a*).

Whitehead (1976) has discussed the possibility that constraints between rate constants could limit or even suppress the kinetic co-operativity of several enzyme systems. In particular, he has examined the possibility that, for several enzyme forms from which both substrate desorption and catalysis can occur, the ratio of these two rate constants can be invariant, and has coined the term generalized microscopic reversibility to express the view that, under non-equilibrium conditions, some constraints might exist between rate constants that would alter enzyme co-operativity. The first aim of the present paper is to discover some physical bases of generalized microscopic reversibility in both polymeric and monomeric enzymes.

Cornish-Bowden (1976*a*,*b*), Brocklehurst & Cornish-Bowden (1976), Brocklehurst (1977), as well as Albery & Knowles (1976), have discussed quantitatively how an enzyme that obeys simple Michaelis–Menten kinetics can improve its catalytic efficiency during its evolution. Indeed, one may wonder whether there is a connection between the occurrence of generalized microscopic reversibility and the asymptotic approach to a 'catalytic perfection' (Albery & Knowles, 1976) during evolution. To answer this question is the second aim of the present paper.

Theory

The concept of generalized microscopic reversibility for polymeric enzymes

Classical microscopic reversibility is algebraically formulated, in most cases, as a relation occurring between equilibrium constants within a closed loop of a reaction mechanism. This constraint is simply a consequence of the fact that the free-energy change is a state function. As we shall see in this section, it is thermodynamically possible, under certain circumstances, to extend microscopic reversibility to individual rate constants under non-equilibrium conditions of the reactants.

Let us consider first the sequential binding of a substrate on a polymeric enzyme. Each step of the reaction process involves substrate binding, substrate desorption and catalysis (Scheme 1). This situation is obviously a limiting case in which both product concentration is negligible (initial steady-state conditions) and the rate of product desorption is much faster than catalysis. Therefore the concentration of enzyme–product(s) complexes can be neglected. In the general case where substrate release or catalysis requires conformation change, the free energy of activation ($\Delta G^{\ddagger}_{-s}$ and $\Delta G^{\ddagger}_{c}$) of each of these steps can be partitioned into four different contributions (Ricard *et al.*, 1974*a*): an intrinsic component that corresponds to the activation free energy of the step apart from the effects of conformation changes and subunit interactions (this component will correspond to $\Delta G^{\ddagger *}_{-s}$ and $\Delta G^{\ddagger *}_{c}$ in eqn. 1); an intrinsic transconformation component that corresponds to the contribution of a protomeric conformation change on the process, apart from energy contributions of substrate binding, substrate release or catalysis (this component will correspond to $^{-t}\Delta G^{\ddagger *}_{-s}$ and $^{-t}\Delta G^{\ddagger *}_{c}$ in eqn. 1); the contribution of the various types of subunit interactions to the energy associated with substrate release and catalysis without taking into account the conformation change [this contribution will correspond to the two terms $\sum(^{-s}\Delta G^{\ddagger int.})$ and $\sum(^{c}\Delta G^{\ddagger int.})$ in eqn. 1]; the contribution of the various types of subunit interactions to the energy of the conformation change of the polymeric enzyme associated with substrate release and catalysis [this contribution will correspond to the same term $\sum(^{-t}\Delta G^{\ddagger int.})$ in eqn. 1].

Since, in the model of Scheme 1, every desorption step of the substrate is paralleled by a catalytic process leading to the same conformation state of the enzyme, one conformation change at the most is associated with these two processes. If the node compression is justified (which implies that product concentration is very small), and if the simplest version of the transition-state theory can be applied to enzyme systems, one has:

$$\Delta G^{\ddagger}_{-s} = \Delta G^{\ddagger *}_{-s} + (^{-t}\Delta G^{\ddagger *}_{-s}) + \sum(^{-s}\Delta G^{\ddagger int.}) + \sum(^{-t}\Delta G^{\ddagger int.}) \quad (1a)$$

$$\Delta G^{\ddagger}_{c} = \Delta G^{\ddagger *}_{c} + (^{-t}\Delta G^{\ddagger *}_{c}) + \sum(^{c}\Delta G^{\ddagger int.}) + \sum(^{-t}\Delta G^{\ddagger int.}) \quad (1b)$$

The subscript and superscript letters –s and c refer to substrate release and catalysis. Superscript –t refer to the conformation change occurring in the backward (substrate release and catalysis) direction. It is worth noting that, although complicated, this symbolism is the simplest one that takes account of the present situation. Eqns. (1*a*) and (1*b*) are still valid if a binding process occurs without conformation change. One has simply to drop the corresponding terms in eqns. (1*a*) and (1*b*).

Moreover, if m different states are accessible to any protomer within the polymeric enzyme, one will have:

$$\sum(^{-s}\Delta G^{\ddagger int.}) = \sum_{i=1}^{m}\sum_{j=i}^{m} n_{ij}(^{-s}\Delta G^{\ddagger}_{ij}{}^{int.}) \quad (2a)$$

$$\sum(^{c}\Delta G^{\ddagger int.}) = \sum_{i=1}^{m}\sum_{j=i}^{m} n_{ij}(^{c}\Delta G^{\ddagger}_{ij}{}^{int.}) \quad (2b)$$

$$\sum(^{-t}\Delta G^{\ddagger int.}) = \sum_{i=1}^{m}\sum_{j=i}^{m} n_{ij}(^{-t}\Delta G^{\ddagger}_{ij}{}^{int.}) \quad (2c)$$

where n_{ij} represent the numbers of interactions between state i and state j within the polymer. The intrinsic energy terms (those marked with a star in eqns. 1*a* and 1*b*) allow one to define an intrinsic rate constant for each process (substrate release and catalysis). One has thus:

$$k^{*}_{-s} = \frac{k_B T}{h}\exp\{-[\Delta G^{\ddagger *}_{-s} + (^{-t}\Delta G^{\ddagger *}_{-s})]/RT\} \quad (3a)$$

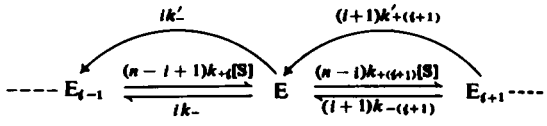
$$k^{*}_{c} = \frac{k_B T}{h}\exp\{-[\Delta G^{\ddagger *}_{c} + (^{-t}\Delta G^{\ddagger *}_{c})]/RT\} \quad (3b)$$

where k_B, h, T and R are the Boltzmann constant, the Planck constant, the absolute temperature and the gas constant. Similarly, from the interaction energy terms between two identical conformations A and A in eqns. (1*a*) and (1*b*) one obtains the definition of interaction coefficients. Thus:

$$(^{-s}\alpha_{AA}) = \exp\{-(^{-s}\Delta G^{\ddagger int.}_{AA})/RT\} \quad (4a)$$

$$(^{c}\alpha_{AA}) = \exp\{-(^{c}\Delta G^{\ddagger int.}_{AA})/RT\} \quad (4b)$$

$$(^{-t}\alpha_{AA}) = \exp\{-(^{-t}\Delta G^{\ddagger int.}_{AA})/RT\} \quad (4c)$$



Scheme 1. *Sequential binding of a substrate on a multimeric enzyme*

n represents the number of steps. The value of each rate constant is multiplied by a corresponding statistical factor.

and one could define in the same way the interaction coefficients between any types of conformation. All the interaction coefficients are dimensionless parameters that equal 1 when the corresponding interaction energies are zero. Having defined in thermodynamic terms the three intrinsic rate constants and all the interaction coefficients, eqns. (1*a*) and (1*b*) become equivalent to:

$$k_{-s} = k^*_{-s} \prod_{i=1}^{m} \prod_{j=i}^{m} \{(^{s}\alpha_{ij})(^{t}\alpha_{ij})\}^{n_{ij}} \qquad (5a)$$

$$k_c = k^*_c \prod_{i=1}^{m} \prod_{j=i}^{m} \{(^{c}\alpha_{ij})(^{t}\alpha_{ij})\}^{n_{ij}} \qquad (5b)$$

In this form, it is clear that the intrinsic rate constants correspond to what the rate constants for substrate release and catalysis would be if the enzyme did not display any subunit interaction. Similarly, the α coefficients express the effect of various types of subunit interactions on the rate of substrate release and catalysis, as well as on the conformation changes associated with these processes.

A very interesting situation is observed if subunit interactions do not affect the final geometry of the active site, but simply increase or decrease the rate of its conformational transition, thus exerting only an indirect effect on the rate constants of substrate release and catalysis. Then:

$$\sum (^{s}\Delta G^{\ddagger int.}) = 0 \qquad (6a)$$

$$\sum (^{c}\Delta G^{\ddagger int.}) = 0 \qquad (6b)$$

which implies:

$$\prod_{i=1}^{m} \prod_{j=i}^{m} (^{s}\alpha_{ij})^{n_{ij}} = 1 \qquad (7a)$$

$$\prod_{i=1}^{m} \prod_{j=i}^{m} (^{c}\alpha_{ij})^{n_{ij}} = 1 \qquad (7b)$$

and eqns. (5*a*) and (5*b*) become:

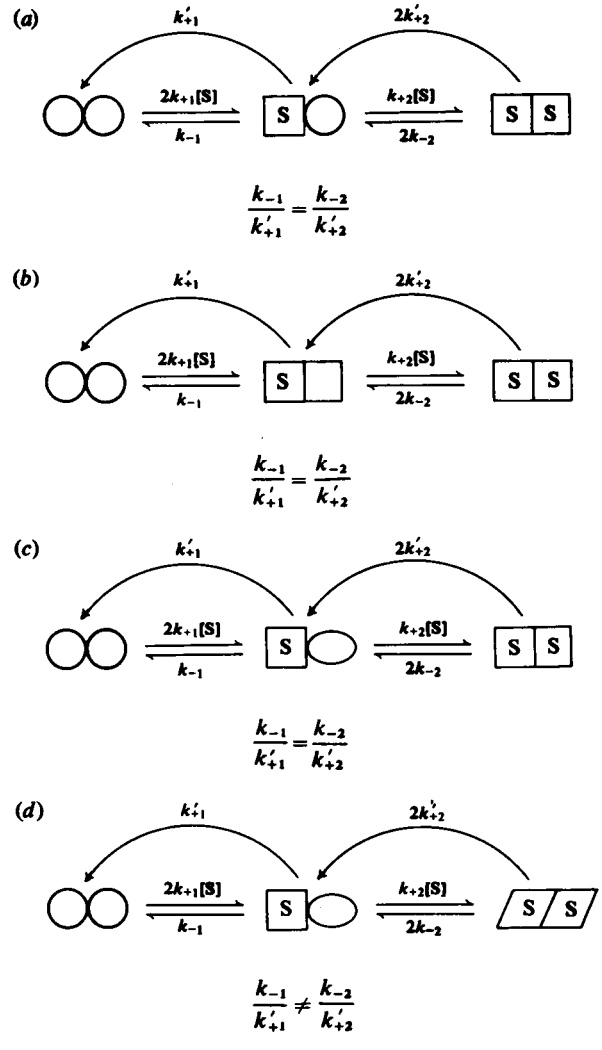
$$k_{-s} = k^*_{-s} \prod_{i=1}^{m} \prod_{j=i}^{m} (^{t}\alpha_{ij})^{n_{ij}} \qquad (8a)$$

$$k_c = k^*_c \prod_{i=1}^{m} \prod_{j=i}^{m} (^{t}\alpha_{ij})^{n_{ij}} \qquad (8b)$$

If these expressions are applied to the model of Scheme 1, one sees that:

$$\frac{k'_{+i}}{k_{-i}} = \frac{k'_{+(i+1)}}{k_{-(i+1)}} \text{ (for } i = 1,2, \ldots . n-1) \qquad (9)$$

which corresponds precisely to the formulation of generalized microscopic reversibility. Therefore the condition of generalized microscopic reversibility is compulsory for polymeric enzymes if subunit interactions are such that eqns. (6) and (8) are fulfilled. It is then of cardinal importance to discuss the physical significance of eqns. (6) and (8) for some simple models of subunit interactions.



Scheme 2. *Generalized microscopic reversibility and some models of subunit interactions for dimeric enzymes* (*a*) Simple sequential model. (*b*) Fully concerted sequential model. (*c*) Partially concerted sequential model with three states accessible to a protomer. (*d*) Partially concerted sequential model with four different states accessible to a protomer.

Scheme 2 shows four of these models. Model 1 can be defined as simple sequential (Koshland *et al.*, 1966; Ricard *et al.*, 1974*a*). Two states (the circle and the square) are accessible to the protomers. The association of the subunits is assumed to be loose enough so that the occupancy of the first site by the substrate has no effect on the geometry of the other subunit. Application of the above conditions to this model shows that generalized microscopic revers-

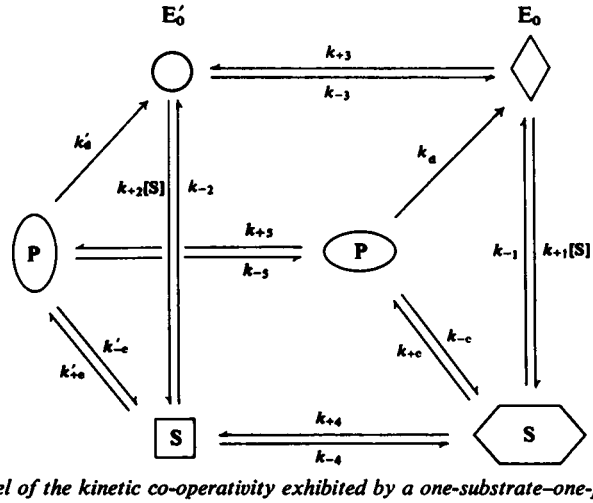
ibility is compulsory. Model II is defined as fully concerted (Ricard *et al.*, 1974*a*). It is an 'all-or-none' model. The subunits are so tightly associated that any change in the conformation of a subunit is followed by a similar conformation change in the other. As with model I, two states are accessible to the protomers. Again, generalized microscopic reversibility must, of necessity, apply to this model. Model III is intermediary between the two previous ones. Three states are accessible to the protomers and generalized microscopic reversibility must apply. In model IV four states are accessible to the protomers. Contrary to the previous Schemes the geometry of the enzyme–substrate complex is variable depending on whether the enzyme is half-saturated or fully saturated by its substrate. Whereas eqns. (6) and (7) apply to models I, II and III they do not to model IV, and therefore the requirement for generalized microscopic reversibility is not satisfied in this case. This conclusion can be formulated in two different ways. (1) When, in a reaction sequence, the conformation of a protomer saturated by its substrate is not changed by different subunit interactions, generalized microscopic reversibility must, of necessity, apply. (2) If generalized microscopic reversibility applies within a reaction sequence, then only one conformation state is accessible to any protomer that has already bound its substrate.

Models of Scheme 3 are based on the concept of induced fit in the strict sense given by Koshland (1970) (the enzyme has to change its conformation to bind the substrate). They use the node compression already present in Scheme 1 and widely used in other papers (see Whitehead, 1976). Moreover the same type of reasoning would apply if conformation change and substrate binding were occurring as two distinct steps, as considered in the model proposed by Monod *et al.* (1965).

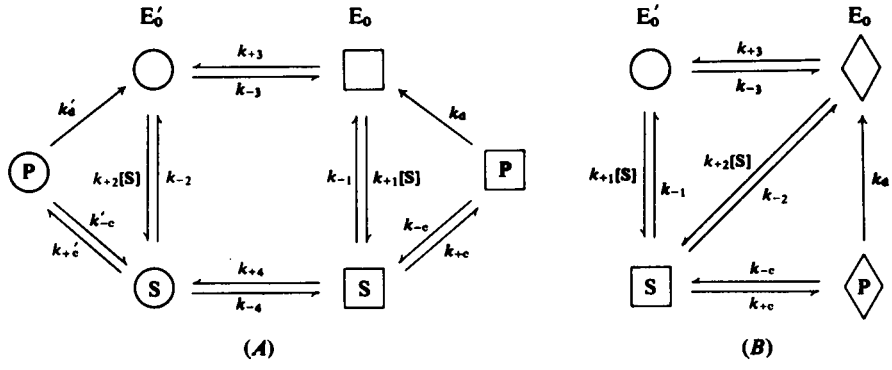
Generalized microscopic reversibility for monomeric enzymes

Let us consider now the case of a monomeric enzyme occurring in two conformation states (the circle and the rhombus). Substrate is assumed to bind to the conformation state inducing a conformation change. Catalysis and product desorption are also assumed to be accompanied by conformation changes of the enzyme. The step of product desorption is set as irreversible because the model is analysed under initial steady-state conditions. All the enzyme forms are assumed to be interconvertible (Scheme 3). This model, which I shall designate by the term 'general', can lead to two interesting special cases (Scheme 4). The slow-transition model differs from the 'general model' in that it involves only two different conformation states of the enzyme and no enzyme–product interconversion; co-operativity would appear as the consequence of a shift of a pre-existing equilibrium. The simple mnemonical model, on the other hand, does involve induced-fit processes (in the strict meaning used by Koshland, 1970), but stresses two important ideas: only one geometry of the active site is able to effect catalysis; secondly, the product stabilizes one of the conformation states (enzyme memory). Obviously, the slow-transition model and the simple mnemonical model do not derive from each other because they use different basic concepts, but they both derive from the same 'general model'.

The 'general model' of Scheme 3 can be symbolically expressed as shown in Scheme 5. The free-energy



Scheme 3. *'General' model of the kinetic co-operativity exhibited by a one-substrate–one-product monomeric enzyme*



Scheme 4. *The slow-transition model (a) and the mnemonical model (b) for a one-substrate, one-product monomeric enzyme*

difference within each transition-state pair $\delta\Delta G_{\overline{es}}$, $\delta\Delta G_{\overline{ex}}$, $\delta\Delta G_{\overline{ep}}$ (where the lower case indices $\overline{es}$ etc. refer to transition states) along the reaction co-ordinate allows the definition of a destabilization coefficient (‡):

$$\delta_{\overline{es}} = e^{-\delta\Delta G_{\overline{es}}/RT} \qquad (10a)$$

$$\delta_{\overline{ex}} = e^{-\delta\Delta G_{\overline{ex}}/RT} \qquad (10b)$$

$$\delta_{\overline{ep}} = e^{-\delta\Delta G_{\overline{ep}}/RT} \qquad (10c)$$

All of these destabilization coefficients are dimensionless and equal to 1 if the corresponding free-energy difference is zero. In addition to classical microscopic reversibility, which imposes:

$$\frac{k_{+1}}{k_{-1}}\frac{k_{+4}}{k_{-4}} = \frac{k_{+3}}{k_{-3}}\frac{k_{+2}}{k_{-2}} \qquad (11a)$$

$$\frac{k_{+c}}{k_{-c}}\frac{k_{+5}}{k_{-5}} = \frac{k_{+4}}{k_{-4}}\frac{k'_{+c}}{k'_{-c}} \qquad (11b)$$

the following conditions can easily be derived from Scheme 5:

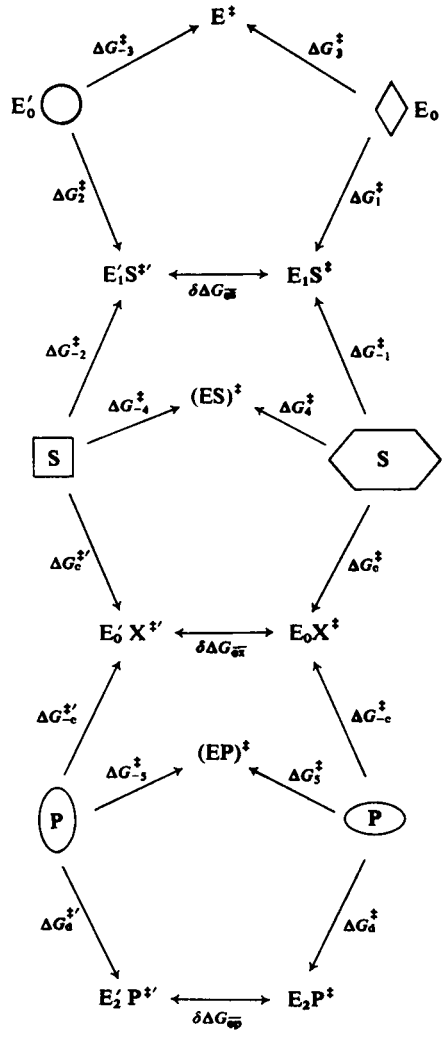
$$\frac{k_{+1}}{k_{+2}}\delta_{\overline{es}} = \frac{k_{+3}}{k_{-3}} \qquad (12a)$$

$$\frac{k_{-1}}{k_{-2}}\frac{\delta_{\overline{es}}}{\delta_{\overline{ex}}} = \frac{k_{+c}}{k'_{+c}} \qquad (12b)$$

$$\frac{k_{-1}}{k_{-2}}\delta_{\overline{es}} = \frac{k_{+4}}{k_{-4}} \quad \text{and} \quad \frac{k_{+c}}{k'_{+c}}\delta_{\overline{ex}} = \frac{k_{+4}}{k_{-4}} \qquad (12c)$$

$$\frac{k_{-c}}{k'_{-c}}\frac{\delta_{\overline{ex}}}{\delta_{\overline{ep}}} = \frac{k_d}{k'_d} \qquad (12d)$$

$$\frac{k_{-c}}{k'_{-c}}\delta_{\overline{ex}} = \frac{k_{+5}}{k_{-5}} \quad \text{and} \quad \frac{k_d}{k'_d}\delta_{\overline{ep}} = \frac{k_{+5}}{k_{-5}} \qquad (12e)$$



Scheme 5. *Activation free energies connecting the various enzyme forms in the 'general' model*

$\delta\Delta G_{\overline{es}}$, $\delta\Delta G_{\overline{ex}}$, $\delta\Delta G_{\overline{ep}}$ represent the free-energy differences between the various transition states. The free enzyme forms E_0 and E'_0 are assumed to be complementary to the transition states $X^{\ddagger}$ and $X^{\ddagger\prime}$ respectively.

Except for the expression involving k_{+5} and k_{-5} the above equations obviously apply also to the slow-transition model. Two interesting conclusions can be drawn from eqns. (12): first, if the free-energy difference between the first two transition states is equal to the free-energy change between the two free enzyme forms ($\delta_{\overline{cs}} = k_{+3}/k_{-3}$), the two on-constants k_{+1} and k_{+2} will be equal; secondly, if the free-energy difference within each transition-state pair is held constant along the reaction co-ordinate, eqns. (12*b*) and (12*d*) become:

$$\frac{k_{-1}}{k_{-2}} = \frac{k_{+c}}{k'_{+c}} \qquad (13a)$$

$$\frac{k_{-c}}{k'_{-c}} = \frac{k_d}{k'_d} \qquad (13b)$$

and, as shown below, are algebraic formulations of generalized microscopic reversibility. The thermodynamic conditions that generate these equalities are thus:

$$\delta\Delta G_{\overline{cs}} = \delta\Delta G_{\overline{cx}} = \delta\Delta G_{\overline{cp}} \qquad (14)$$

In Scheme 4 it has been assumed that the two free enzyme forms E_0 and E'_0 are complementary to the corresponding transition states $X^{\ddagger}$ and $X^{\ddagger\prime}$, whereas they are more or less 'strained' (Jencks, 1975) in all the other enzyme–ligand complexes. A typical energy profile that fulfils these conditions of generalized microscopic reversibility is shown in Fig. 1. These energy profiles must take account of the Hammond postulate (Jencks, 1969), which implies that the transition states of the fastest and thus energetically more-favoured process will occur earlier along the reaction co-ordinate. This postulate imposes a shifting of the transition states of each pair ($d_{\overline{s}}$, $d_{\overline{x}}$, $d_{\overline{p}}$ in Fig. 1) along the reaction co-ordinate. If, for instance, E_0 is the fastest enzyme form, the transition state $S^{\ddagger}$ will resemble the unstrained substrate S more closely than the other transition state $S^{\ddagger\prime}$. A similar situation will apply to the other transition states $X^{\ddagger}$ or $P^{\ddagger}$. Moreover, the reaction profile of Fig. 1 takes account of the well-known idea that the free-energy difference is lower in the transition states than in the enzyme–substrate and enzyme–product strained complexes.

It is commonly considered (Wolfenden, 1969; Secemski & Lienhard, 1971; Lienhard *et al.*, 1972) that the free enzyme has a higher affinity for the transition state, $X^{\ddagger}$, than for the substrate or the product. This is based on the assumption made long ago by Haldane (1930) that the geometry of the active site is more complementary to the transition state $X^{\ddagger}$ than to the substrate itself. This statement gives support to the strain theories (Lumry, 1959) of enzyme action, which postulate that the driving force for overcoming the energy barrier comes in part from the enzyme itself, which attains its unstrained conformation at the top of the barrier. It would then be of great interest to express eqn. (14) in terms of binding energies of the various transition states to the enzyme. This would allow one to link generalized microscopic reversibility with a more or less tight

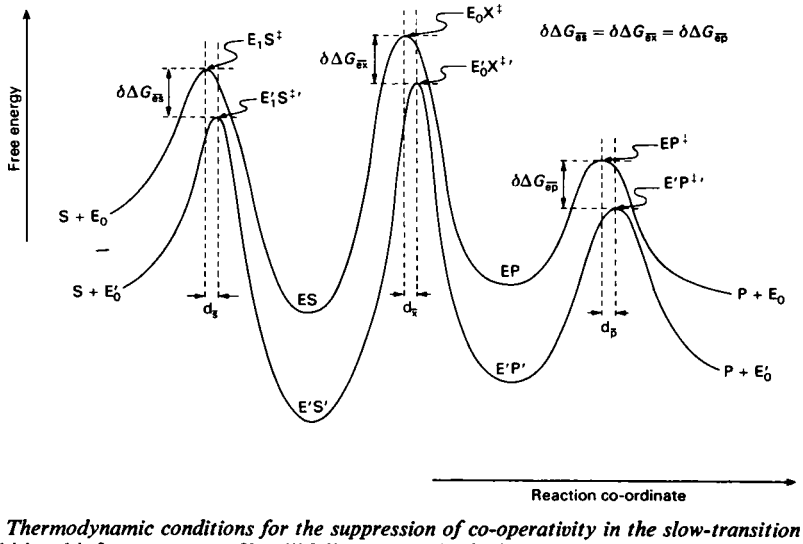


Fig. 1. *Thermodynamic conditions for the suppression of co-operativity in the slow-transition model*
The enzyme exhibiting this free-energy profile will follow generalized microscopic reversibility because $\delta\Delta G_{\overline{cs}} = \delta\Delta G_{\overline{cx}} = \delta\Delta G_{\overline{cp}}$.

complementarity between the free enzyme and the transition states.

This new formulation can be set up with the help of 'thermodynamic boxes' (Lienhard *et al.*, 1972), as shown in Scheme 6. It must be understood that the boxes represent ideal thermodynamic situations and not reaction processes that actually occur during substrate binding and catalysis. The basic idea of the Schemes is simply to split the free-energy change between the initial state and any enzyme–transition state complex into three ideal contributions: (*a*) the free-energy change required to bring the initial substrate to the level of any transition state (for example $\Delta G^{\ddagger}_{\bar{s}\bar{s}}$, $\Delta G^{\ddagger}_{\bar{s}\bar{x}}$, $\Delta G^{\ddagger}_{\bar{s}\bar{p}}$ in Scheme 6), (*b*) the free-energy change required to bring the enzyme from its initial state to the state complementary to any transition state (for example ΔG^{T}_{01}, ΔG^{T}_{02} in Scheme 6), and (*c*) the free binding energy of any transition state to the complementary enzyme form ($-\Delta G^{B}_{\bar{s}}$, $-\Delta G^{B}_{\bar{x}}$, $-\Delta G^{B}_{\bar{p}}$ in Scheme 6). The same set of definitions will obviously apply to the two reaction profiles of Fig. 1; moreover, as in strain theory (Jencks, 1975), the two free enzyme forms E_0 and E_0' are assumed to be complementary to $X^{\ddagger}$ and $X^{\ddagger\prime}$ respectively. In this way one has:

$$\Delta G_1^{\ddagger} = -\Delta G^{B}_{\bar{s}} + \Delta G^{T}_{01} + \Delta G^{\ddagger}_{\bar{s}\bar{s}} = \varepsilon_{\bar{s}\bar{s}} \qquad (15a)$$

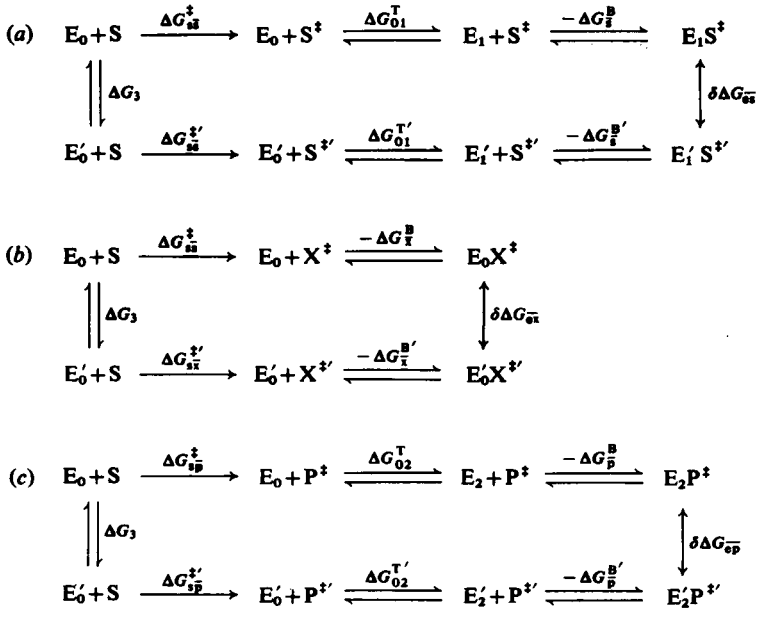
$$\Delta G_1^{\ddagger} + \Delta G_c^{\ddagger} - \Delta G^{\ddagger}_{-1} = -\Delta G^{B}_{\bar{x}} + \Delta G^{\ddagger}_{\bar{s}\bar{x}} = \varepsilon_{\bar{s}\bar{x}} \qquad (15b)$$

$$\Delta G_1^{\ddagger} + \Delta G_c^{\ddagger} + \Delta G_d^{\ddagger} - \Delta G^{\ddagger}_{-1} - \Delta G^{\ddagger}_{-c} = \Delta G^{B}_{\bar{p}} + \Delta G^{T}_{02} + \Delta G^{\ddagger}_{\bar{s}\bar{p}} = \varepsilon_{\bar{s}\bar{p}} \qquad (15c)$$

These expressions imply that part of the free energy required to go from the initial to any transition state along the reaction co-ordinate is 'paid for' by the corresponding binding energy. The 'extra costs' that are not 'paid for' by these binding energies are the ε terms of eqns. (15). These 'extra costs', ε, are thus the algebraic sum of two terms; the free energy of activation required to reach any transition state from the initial state, and the apparent, or real, binding energy of the transition states to the free enzyme. Since, as shown in the thermodynamic boxes of Scheme 6, the free enzyme cannot be complementary to the substrate- and product-transition states, the productive binding process thus requires the proper conformation change of the active site (energy terms ΔG^{T}_{01} and ΔG^{T}_{02}). The apparent binding energies of the substrate- and product-transition states are thus:

$$-\Delta\tilde{G}^{B}_{\bar{s}} = -\Delta G^{B}_{\bar{s}} + \Delta G^{T}_{01} \qquad (16a)$$

$$-\Delta\tilde{G}^{B}_{\bar{p}} = -\Delta G^{B}_{\bar{p}} + \Delta G^{T}_{02} \qquad (16b)$$



Scheme 6. *Thermodynamic boxes allowing the formulation of generalized microscopic reversibility in terms of binding energies of the various transition states by using the 'general' model of Scheme* 3

The enzyme forms E_0 and E_0' are assumed to be complementary to the transition states $X^{\ddagger}$ and $X^{\ddagger\prime}$ respectively (for further details see the text).

and represent what is left after part of the real binding energy has been used to produce the enzyme conformation change.

The condition represented by eqn. (14) that implies the occurrence of generalized microscopic reversibility can be rewritten as:

$$\Delta G_c^{\ddagger} - \Delta G_{-1}^{\ddagger} = \Delta G_c^{\ddagger\prime} - \Delta G_{-2}^{\ddagger} \qquad (17a)$$

$$\Delta G_d^{\ddagger} - \Delta G_{-c}^{\ddagger} = \Delta G_d^{\ddagger\prime} - \Delta G_{-c}^{\ddagger\prime} \qquad (17b)$$

Algebraic manipulation of eqns. (15) gives:

$$\Delta G_c^{\ddagger} - \Delta G_{-1}^{\ddagger} = \varepsilon_{s\bar{x}} - \varepsilon_{s\bar{s}} \qquad (18a)$$

$$\Delta G_d^{\ddagger} - \Delta G_{-c}^{\ddagger} = \varepsilon_{s\bar{p}} - \varepsilon_{s\bar{x}} \qquad (18b)$$

In the same way one could show that:

$$\Delta G_c^{\ddagger\prime} - \Delta G_{-2}^{\ddagger} = \varepsilon'_{s\bar{x}} - \varepsilon'_{s\bar{s}} \qquad (19a)$$

$$\Delta G_d^{\ddagger\prime} - \Delta G_{-c}^{\ddagger\prime} = \varepsilon'_{s\bar{p}} - \varepsilon'_{s\bar{x}} \qquad (19b)$$

and eqns. (17) can be rewritten as:

$$\varepsilon_{s\bar{x}} - \varepsilon_{s\bar{s}} = \varepsilon'_{s\bar{x}} - \varepsilon'_{s\bar{s}} \qquad (20a)$$

$$\varepsilon_{s\bar{p}} - \varepsilon_{s\bar{x}} = \varepsilon'_{s\bar{p}} - \varepsilon'_{s\bar{x}} \qquad (20b)$$

Therefore generalized microscopic reversibility requires that the difference between any two consecutive 'extra costs' of energy along one reaction co-ordinate be the same as the corresponding difference on the other reaction co-ordinate. This is shown in the free-energy profiles of Fig. 2.

As stated above, the slow-transition model (Ainslie *et al.*, 1972) implies that the enzyme–product complexes are not interconvertible and that the enzyme does not change its conformation along the reaction co-ordinate. The thermodynamic equilibria of Scheme 6 then contract to those of Scheme 7. As before, the conditions that allow eqns. (13) to be fulfilled are still eqns. (14), and the necessary condition for generalized microscopic reversibility is still represented by eqns. (20). The physical significance of these equations is unaffected in the slow-transition model, the only difference with respect to the 'general model' being that the apparent binding energies have been replaced by real binding energies since binding energy is not used (Scheme 7) to promote conformation change of the enzyme.

The mnemonical model is another limiting case of the 'general model' in which one of the free enzyme forms must be complementary to the transition state $X^{\ddagger}$, whereas the other one must be complementary to the product and to its transition state, $P^{\ddagger}$. The thermodynamic equilibria of Scheme 6 then contract to those shown in Scheme 8. A typical energy profile of a mnemonical enzyme is shown in Fig. 3. Clearly eqns. (14) cannot apply to this situation. An interesting condition that, as will be seen below, controls the kinetic co-operativity of a mnemonical

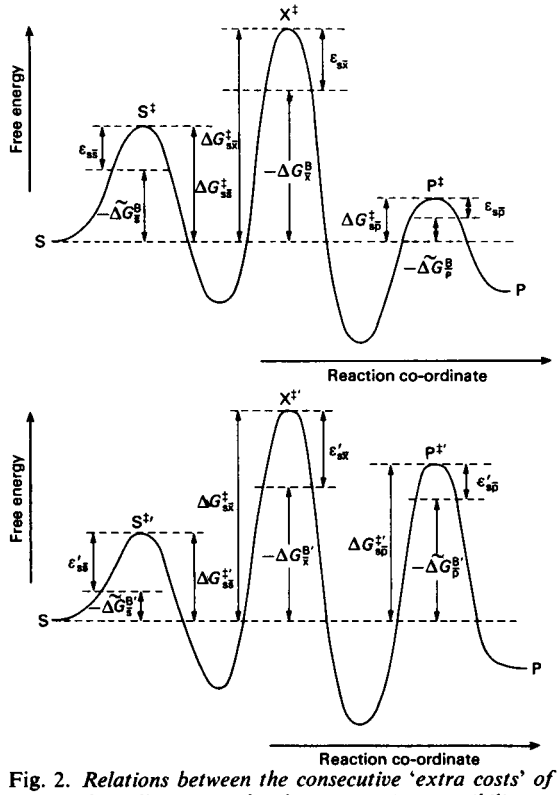


Fig. 2. *Relations between the consecutive 'extra costs' of energy that allow generalized microscopic reversibility to be fulfilled*

The model considered is the 'general' model of Scheme 3. It is noteworthy that the conditions:

$$\varepsilon_{s\bar{x}} - \varepsilon_{s\bar{s}} = \varepsilon'_{s\bar{x}} - \varepsilon'_{s\bar{s}}$$

$$\varepsilon_{s\bar{p}} - \varepsilon_{s\bar{x}} = \varepsilon'_{s\bar{p}} - \varepsilon'_{s\bar{x}}$$

are fulfilled (for further details see the text).

enzyme can easily be derived from the 'thermodynamic boxes' of Scheme 8. One has:

$$\Delta G_1^{\ddagger} = \Delta G_{s\bar{s}}^{\ddagger} + \Delta G_{01}^{T} - \Delta G_s^{B} \qquad (21a)$$

$$\Delta G_2^{\ddagger} = \Delta G_{s\bar{s}}^{\ddagger\prime} + \Delta G_{01}^{T\prime} - \Delta G_s^{B\prime} \qquad (21b)$$

and

$$\Delta G_1^{\ddagger} + \delta\Delta G_{\bar{es}} = \Delta G_3 + \Delta G_2^{\ddagger} \qquad (22)$$

therefore, if:

$$\delta\Delta G_{\bar{es}} > \Delta G_3 \qquad (23)$$

then:

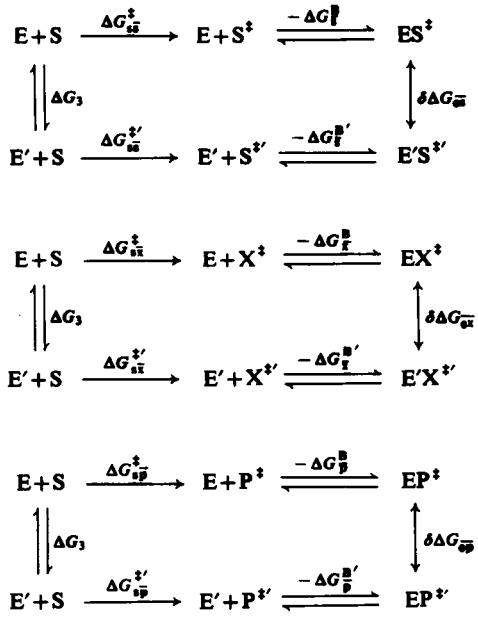
$$\Delta G_2^{\ddagger} > \Delta G_1^{\ddagger} \qquad (24)$$

and conversely, if:

$$\delta\Delta G_{\bar{es}} < \Delta G_3 \qquad (25)$$

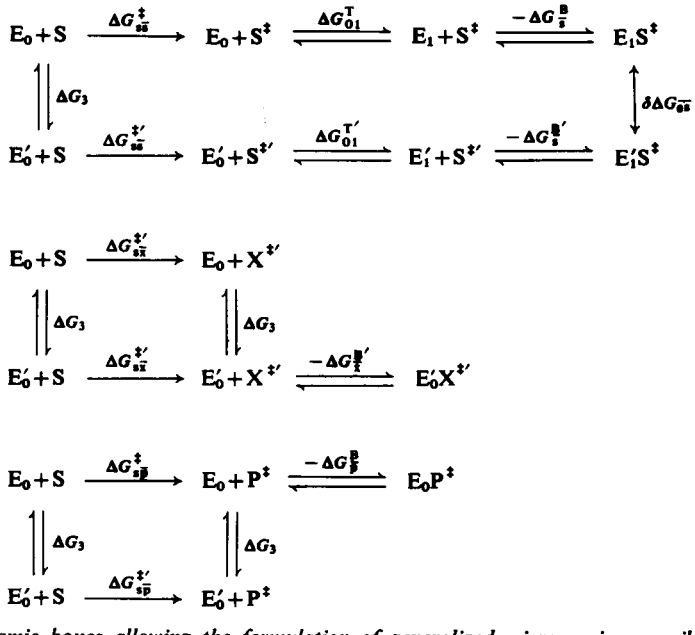
then:

$$\Delta G_2^{\ddagger} < \Delta G_1^{\ddagger} \qquad (26)$$



Scheme 7. *Thermodynamic boxes allowing the formulation of generalized microscopic reversibility in terms of binding energies of the various transition states by using the slow-transition model of Scheme* 4(*a*)

The conformation of the two enzyme forms E and E′ are assumed not to change during the binding of the transition states. See the text for further details.



Scheme 8. *Thermodynamic boxes allowing the formulation of generalized microscopic reversibility in terms of binding energies of the various transition states by using the mnemonical model of Scheme* 4(*b*)

The free enzyme E_0' is assumed to be complementary to the transition state $X^{\ddagger\prime}$, whereas the other enzyme form E_0 is more or less complementary to both product P and its transition state $P^{\ddagger}$. See the text for further details.

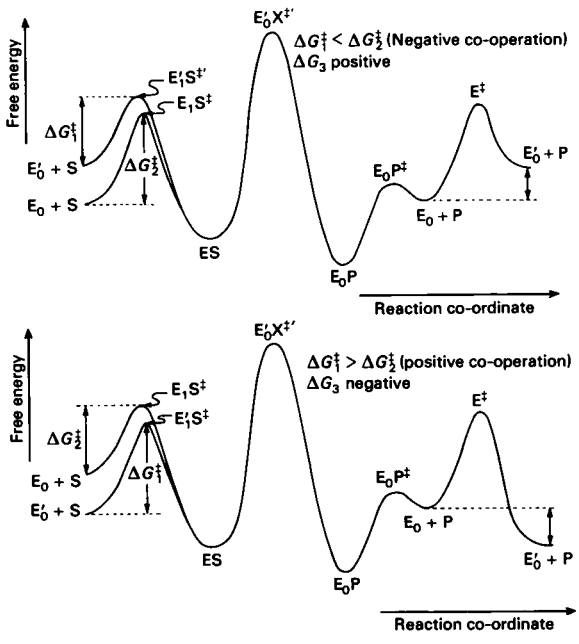


Fig. 3. *Thermodynamic conditions that control positive or negative co-operation for a mnemonical enzyme*
This Figure clearly illustrates the view that the sign of the free-energy change of the enzyme's conformational transition (ΔG_3) is linked with the sign of the co-operation.

The significance of these results will be discussed in the next section. It is interesting to note that the respective values of $\Delta G_2^{\ddagger}$ and $\Delta G_1^{\ddagger}$ are linked with the sign of the free-energy change of the conformational transition of the free enzyme. This is a consequence of the Hammond postulate and of the view that a transition state $ES^{\ddagger}$ must have a structure midway between those of E + S and ES. Therefore the free-energy difference between the two transition states $ES^{\ddagger}$ and $E'S^{\ddagger}$ must be smaller than that between E_0 and E'_0. This is exemplified in Fig. 3. If:

$$\Delta G_1^{\ddagger} > \Delta G_2^{\ddagger} \tag{27}$$

then:

$$\Delta G_3 < 0 \tag{28}$$

and if:

$$\Delta G_1^{\ddagger} < \Delta G_2^{\ddagger} \tag{29}$$

then:

$$\Delta G_3 > 0 \tag{30}$$

Indeed these conclusions would not hold if substrate binding and induced transconformation occurred in two distinct steps instead of one (Ricard *et al.*, 1977).

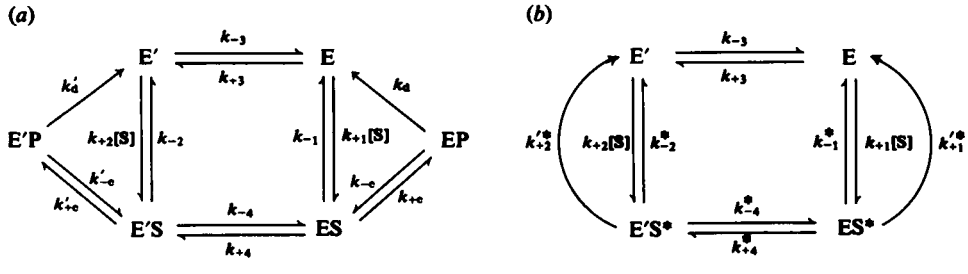
Constraint between rate constants and the kinetic co-operativity of enzymes

As already suggested by Whitehead (1976) the fact that generalized microscopic reversibility holds may reduce the degree of some of the rate equations describing the kinetic behaviour of polymeric enzymes. This point has already been discussed by Whitehead (1976) and need not be developed any further here. The discussion will be centred mostly on the kinetic co-operativity that may, or may not, be generated by the slow-transition model (Ainslie *et al.*, 1972).

This model is formally represented in Scheme 9(*a*). From graph theory, a node compression (Whitehead, 1976; Berge, 1957) can be effected in this reaction scheme so that it can be expressed as shown in Scheme 9(*b*). In this condensed version of the slow-transition model:

$$[ES^*] = [ES] + [EP] \tag{31a}$$

$$[E'S^*] = [E'S] + [E'P] \tag{31b}$$



Scheme 9. *Node compression in the slow-transition model*
(*a*) Slow-transition model. (*b*) Slow-transition model after node compression.

and the k^* are apparent rate constants:

$$k^*_{-1}=\frac{k_{-1}(k_d+k_{-c})}{k_d+k_{+c}+k_{-c}} \quad k^*_{-2}=\frac{k_{-2}(k'_d+k'_{-c})}{k'_d+k'_{+c}+k'_{-c}} \quad (32a)$$

$$k'^*_1=\frac{k_d k_{+c}}{k_d+k_{+c}+k_{-c}} \quad k'^*_2=\frac{k'_d k'_{+c}}{k'_d+k'_{+c}+k'_{-c}} \quad (32b)$$

$$k^*_{+4}=\frac{k_{+4}(k_d+k_{-c})}{k_d+k_{+c}+k_{-c}} \quad k^*_{-4}=\frac{k_{-4}(k'_d+k'_{-c})}{k'_d+k'_c+k'_{-c}} \quad (32c)$$

By using the method of Botts & Morales (1953) one can determine the non-trivial condition that contracts the slow-transition model to one that predicts Michaelis–Menten kinetics. This condition is:

$$\frac{k'^*_{+2}}{k^*_{-2}}=\frac{k'^*_{+1}}{k^*_{-1}} \quad (33)$$

which corresponds to generalized microscopic reversibility. Eqn. (33) can be rewritten as:

$$\frac{k_{+c}k_d}{k_{-1}(k_{-c}+k_d)}=\frac{k'_c k'_d}{k_{-2}(k'_{-c}+k'_d)} \quad (34)$$

If the condition represented by eqn. (34) is satisfied the slow-transition model cannot predict any co-operativity. It is of interest to note that eqns. (13*a*) and (13*b*) are precisely equivalent to this condition.

In the case of the mnemonical model, the departure from Michaelis–Menten behaviour can be quantitatively expressed by what we have defined (Ricard *et al.*, 1974*b*) as the extent of co-operation, Γ, between the two free enzyme forms, namely:

$$\Gamma=\frac{2k_{+3}}{k_{+1}k_{+2}}(k_{+2}-k_{+1}) \quad (35)$$

and the kinetic co-operativity is suppressed if $k_{+1}=k_{+2}$. Conversely, kinetic co-operativity is negative if $k_{+1}>k_{+2}$ and positive if $k_{+1}<k_{+2}$.

Kinetic co-operativity of monomeric enzymes and their evolution toward 'catalytic perfection'

Albery & Knowles (1976) have recently developed a formalism that allows one to express quantitatively the 'catalytic perfection' of a monomeric enzyme. The occurrence of 'catalytic perfection' implies that substrate and product diffuse freely to the active site and that the free-energy differences, within the internal states, between the maxima and the minima along the reaction co-ordinate are very small. If so, the rate constants associated with the internal states will be kinetically insignificant.

In the slow-transition model, thermodynamics requires that:

$$\frac{k_{+1}k_{+c}k_d}{k_{-1}k_{-c}k_{-d}}=\frac{k_{+2}k'_{+c}k'_d}{k_{-2}k'_{-c}k'_{-d}} \quad (36)$$

If, as is assumed in the concept of 'catalytic perfection', substrate and product diffuse freely to the active site, the above expression contracts to:

$$\frac{k_{+c}k_d}{k_{-1}k_{-c}}=\frac{k'_{+c}k'_d}{k_{-2}k'_{-c}} \quad (37)$$

The concept of 'catalytic perfection' applied to two free enzyme forms implies that:

$$\frac{k_{-1}}{k_{+1}k_{+c}}=\frac{k_{-2}}{k_{+2}k'_{+c}} \quad (38)$$

If k_{+1} and k_{+2} are both diffusion-controlled, eqn. (38) contracts to:

$$\frac{k_{-1}}{k_{+c}}=\frac{k_{-2}}{k'_{+c}} \quad (39)$$

Combining eqns. (39) and (37) precisely gives eqn. (34), the condition that suppresses kinetic co-operativity.

The important conclusion is thus that a monomeric enzyme obeying the slow-transition model cannot simultaneously display a kinetic co-operativity and be catalytically perfect. The same conclusion applies to a monomeric enzyme obeying the simple mnemonical model (Fig. 3). If such an enzyme had reached 'catalytic perfection', the two rate constants k_{+1} and k_{+2} would be both diffusion-controlled and thus have identical values leading to a Γ value equal to zero. Therefore the regulatory power of a monomeric enzyme is exerted at the expense of its catalytic efficiency.

Discussion

The fact that subunit interaction within a polymeric enzyme can increase or decrease the rate of a given chemical process can be assigned to two different types of events: a deformation of the active site that makes substrate binding, product release and (or) catalysis more, or less, efficient; an effect on the rates of the conformational transitions associated with the above processes. The present work has shown that, if different types of subunit interactions do not induce different geometries of the liganded active site, then generalized microscopic reversibility must apply. The view that there exists only one conformation of the liganded active site within a reaction sequence has been implicitly postulated by Monod *et al.* (1965), Koshland *et al.* (1966), Dalziel (1968) and Ricard *et al.* (1974*a*). It must be stressed again that, if this condition holds, then each catalytic process (with rate constants $k'_{+\alpha}$ and $k'_{+\beta}$ for instance) must be paralleled by substrate dissociation (with rate constants $k_{-\alpha}$ and $k_{-\beta}$) in such a way that $k'_{+\alpha}/k_{-\alpha}=k'_{+\beta}/k_{-\beta}$. As already stated by Whitehead (1976), when this condition is satisfied, the system, although in a steady state, behaves exactly as if it were under pseudo-equilibrium conditions, and therefore the

degree of the steady-state rate equation must be identical with that of the substrate-binding function. Indeed this does not mean that these two expressions are similar. Usually they are not, but they are of the same degree. One of the novel results of this study has been the finding that the physical reason for generalized microscopic reversibility is that different intramolecular constraints within the quaternary structure do not change the absolute geometry of the liganded active site, but only the rate of the conformation changes.

When this concept of generalized microscopic reversibility is applied to a monomeric enzyme that follows the slow-transition model, the enzyme's kinetic co-operativity is lost. The physical reason for the occurrence of a constraint condition between rate constants that suppresses kinetic co-operativity is that the free-energy difference between the corresponding transition states is kept constant along the reaction co-ordinate. This situation implies that the 'extra costs' of energy (required to pass each energy barrier) that are not covered by the corresponding binding energies of the transition states vary in a similar way along the two reaction co-ordinates. Obviously violation of generalized microscopic reversibility does not imply violation of classical microscopic reversibility.

Indeed, the concept of generalized microscopic reversibility cannot be applied to the mnemonical model. The slow-transition model and the mnemonical model are not derived from each other, although they are both derived from a general model of enzyme conformation change. The mnemonical model presents an interesting characteristic. If the kinetic co-operativity of the enzyme is positive, the free-energy change between the form stabilized by the product (the rhombus in Fig. 3) and the other state must be negative. Conversely, if the kinetic co-operativity is negative, the free-energy change between the two free enzyme forms (the rhombus, and the circle) must be positive. In other words, the sign of the kinetic co-operativity is tightly associated with a shift of the transconformation equilibrium between the two free enzyme species. Moreover, the enzyme form that reacts faster with the substrate is the one that is in smaller amount. Indeed this conclusion is valid only if substrate binding and enzyme transconformation occur in only one step.

Albery & Knowles (1976) have discussed on a quantitative basis the idea that, by natural selection, enzymes of higher and higher efficiency have evolved. Since the biosynthesis of an enzyme is expensive in free-energy terms an organism that does the same chemical work at the same rate with less enzyme will have a selective advantage. Albery & Knowles (1976) have stressed the view that the selection tends to adjust the internal states of the free-energy profile in such a way that these internal states become 'kinetically insignificant'. Since the height of the energy barriers corresponding to substrate and product binding cannot be lowered below the diffusion-controlled limit, the proper adjustment of internal states represents the only possibility left to natural selection for increasing the performance of an enzyme. Then, an enzyme will have reached the upper limit of its possible evolution, called 'catalytic perfection', if substrate and product freely diffuse to the active site and if the internal states are 'kinetically insignificant'. Albery & Knowles (1976) have claimed that triose phosphate isomerase has very nearly reached this 'catalytic perfection'.

If a monomeric enzyme follows the slow-transition model, its 'catalytic perfection' implies of necessity the occurrence of generalized microscopic reversibility. Therefore, the regulatory power of a monomeric enzyme is 'paid for' by the catalytic efficiency of the enzyme. The same conclusion obviously applies to a mnemonical enzyme. If a monomeric enzyme exhibiting memory phenomena has reached 'catalytic perfection' by evolutionary pressure, the bimolecular rate constants k_{+1} and k_{+2} of the mnemonical model will be identical and equal to the diffusion-controlled limit. Therefore the extent of co-operation (Ricard *et al.*, 1974*a*,*b*), Γ, will be equal to zero and the enzyme will not exhibit any kinetic co-operativity. The important idea of this development is thus that a monomeric enzyme cannot simultaneously be efficient and co-operative.

If 'catalytic perfection' implies generalized microscopic reversibility in the slow-transition model it is obvious that the converse is not necessarily true. If we consider for instance the limiting case where the 'extra costs', ε, are equal and constant along the reaction co-ordinate, this represents an obvious selective advantage because, once the enzyme–substrate transition state is formed, the system does not require any additional energy to effect catalysis. Although it may be efficient, this device is not necessarily close to catalytic perfection and still exhibits, of necessity, generalized microscopic reversibility. This reasoning can probably be extrapolated to other situations. In their general evolutionary trend monomeric enzymes obeying the slow-transition model may well exhibit generalized microscopic reversibility long before they have reached 'catalytic perfection'.

The view developed above, that the regulatory power of a monomeric enzyme is antagonistic to its efficiency receives support from several concrete experimental facts. For instance, monomeric wheat-germ hexokinase L_1, which exhibits interesting regulatory properties (Meunier *et al.*, 1974), is a poor catalyst when compared with other phosphotransferases. Similarly, yeast hexokinase in its dissociated monomeric form and its optimum pH (8.5) is a very

efficient enzyme and does not exhibit any atypical behaviour (Colowick, 1973). However, as soon as the pH is decreased to more 'physiological conditions' (pH 6.5–7) its specific activity sharply decreases, but then the monomeric dissociated form presents a regulatory behaviour (Kosow & Rose, 1971; Shill & Neet, 1975). Similar examples might also be found in the literature.

When, in recent years, it became evident that regulatory properties were not strictly limited to polymeric enzymes, but were already present in several monomeric proteins, it was possible to ask the following seemingly anthropomorphic question: why did Nature evolve polymeric regulatory enzymes through neo-Darwinian selection? From the preceding discussion one obtains an obvious and attractive answer: polymeric enzymes represent the simplest device able to exhibit both regulatory power and catalytic efficiency.

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