J. theor. Biol. (1987) **127**, 491–506

Free Energy Differences between Enzyme Bound States

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(*Received* 2 *March* 1987, *and in revised form* 2 *April* 1987)

A theory is presented that describes the free energy difference between the enzyme-substrate (*ES*) and enzyme-product (*EP*) complexes that is expected in enzymes optimized for catalytic efficiency. In such enzymes, the free energy drop between *ES* and *EP* complexes reflects a portion of the chemical potential difference between substrates and products outside the active site under physiological conditions. Qualitative and quantitative predictions of the model are discussed and compared with experimental data. The controversy over the kinetically optimal free energy profile for an enzymatic reaction operating under constraints set forward by Albery & Knowles (1976) is resolved.

In a seminal paper published a decade ago, Albery & Knowles (1976) suggested that "the kinetically significant transition state" of an optimally evolved enzyme "is flanked by kinetically significant intermediates of equal free energy". This simple hypothesis was one of the first to argue that the quantitative behavior of enzymes was functionally optimized (Cleland, 1975; Cornish-Bowden, 1976; Fersht, 1977), and therefore a product of natural selection.

Hypotheses of this type bear directly on an important question in molecular evolution: of the behaviors displayed by biological macromolecules, which ones reflect natural selection, and which ones reflect random drift? (Kimura, 1982; King & Jukes, 1969). A distinction between behaviors that are selected and those that are drifting is, at one level, a distinction between those worth studying in detail and those that are not (Benner *et al.*, 1985). Therefore, functional hypotheses, such as the one proposed by Albery & Knowles, if confirmed by experiment, will have a significant impact both on how enzymes are studied and how the evolution of biological macromolecules is viewed.

The Albery–Knowles hypothesis has been widely discussed (Ashton & Hatch, 1983; Cross, 1981; Senbell & White, 1978; Wilkinson & Rose, 1979, Nageswara Rao & Cohn, 1981; Myers & Boyer, 1984; Lolkema *et al.*, 1986; Cross *et al.*, 1982; Rahil *et al.*, 1982). In the literature, what began (Albery & Knowles, 1976) as a hypothesis for enzymes that had been partially optimized has been interpreted as a general principle of enzymic evolution (Kenyon & Reed 1983; Cook & Cleland, 1981). To the extent that enzymes are kinetically optimized, the hypothesis has been interpreted as predicting that enzyme-bound substrates and enzyme-bound products will have equal Gibbs free energies (Hassett *et al.*, 1982). When the internal equilibrium constant is not displaced towards unity, the enzyme is viewed as lacking an "associated catalytic advantage" (Rahil *et al.*, 1982).

In its current form, the hypothesis of matched internal thermodynamics corresponds to a statement that, for a catalytically optimized enzyme, the "internal equilibrium constant" (the equilibrium constant between enzyme-bound substrates and products, *ES* and *EP*) is unity, regardless of the magnitude of the "external equilibrium constant" (that between substrates and products in solution). In simpler terms, kinetically perfect enzymes, those that have evolved to have the highest rate of turnover, bind substrate and product so that the bound complexes (*ES* and *EP*) have equal free energies.

As expected for so direct and fundamental an assertion, the hypothesis has received much experimental attention in the past decade. Originally, Knowles and his co-workers found that the internal states of triosephosphate isomerase have approximately equal free energies (Knowles & Albery, 1977). Shortly thereafter, Cohn (Nageswara Rao & Cohn, 1981; Nageswara Rao *et al.*, 1979), Rose (Wilkinson & Rose, 1979) and their co-workers reported internal equilibrium constants close to unity in several phosphoryl transferases. These included enzymes that normally catalyze reactions far "downhill" energetically. More recently, the notion that enzymes have internal equilibrium constants close to unity was used to formulate functional hypotheses explaining the stereoselectivity of enzymatic reactions (Benner, 1982*a*,*b*) and as the basis for an analysis of the structure of proteins involved in electron transfer (Rees, 1985).

It appears to be generally true that the energies of enzyme-substrate (*ES*) and enzyme-product (*EP*) complexes are more similar than the energies of substrate (*S*) and product (*P*) free in solution. However, it is also true that many internal equilibrium constants are *not* unity within reported experimental error. For example, the internal equilibrium constant reported for pyruvate kinase is different from unity by approximately an order of magnitude (Stackhouse *et al.*, 1985); the internal equilibrium constant for fructose bisphosphatase appears to be far from unity (Rahil *et al.*, 1982); and the internal equilibrium constants for various dehydrogenases appear to lie between 1:1 to 9:1 (Gutfreund, 1975; Nambiar *et al.*, 1983; Dickinson & Dickenson, 1978).

Based on these results, a new model, the "descending staircase" model, was suggested as an alternative to the "matched internal thermodynamics" model (Stackhouse *et al.*, 1985) to describe the free energy profiles of optimally evolved enzymes. In this model, the internal equilibrium constant reflects a portion of the external drop in chemical potential driving the reaction catalyzed by the enzyme, under the physiological conditions for which the enzyme is adapted.

Several papers have appeared in recent years that either support or modify the hypothesis of matched internal thermodynamics, and a lively controversy has ensued (Cook & Cleland, 1981; Chin, 1983; Rees, 1985). However, it remains a puzzle as to whether enzymes whose internal equilibria are different from unity are enzymes that are not (yet) optimally evolved, whether this hypothesis applies only to certain enzymes, or whether these enzymes should be interpreted as counter-examples to this hypothesis that shed doubt on its validity.

Should the internal equilibrium constant be predictable by a functional theory, the implication would be that natural selection is more powerful than commonly

thought at optimizing the detailed behavior of macromolecules (Benner *et al.*, 1985). Should experimental results not be consistent with the predictions of a functional theory, the implication would be that either kinetic "perfection" as defined by the theory is not a primary goal of natural selection, or that enzymes are unable to achieve kinetic "perfection". Any of these conclusions would be of interest.

However, to date no correct quantitative theory has been published that describes the internal thermodynamics that are expected from a kinetically optimized enzyme. We present a theory in this paper.

Theory

Rate constants are expressed as k_n, where n refers to a designated step in the reaction scheme. Equilibrium constants are expressed as K_n, where n refers to a step in the reaction scheme; equilibrium constants are equal to the ratio of the rate constants for the forward and reverse reactions. S and P indicate the concentrations of substrate and product respectively. β and C are parameters defining a linear free energy relationship of the form

$$k_2 = CK_2^{\beta} \tag{1}$$

where C represents the "intrinsic" rate constant for the reaction (the rate constant where $K_2 = 1$), and β describes the degree to which the transition state for the reaction resembles substrate or product.

We analyze the kinetic model given below

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P.$$

The velocity of an enzymatic reaction is a function of its microscopic kinetic parameters, $v = f(k_1, k_2, k_3, k_{-1}, k_{-2}, k_{-3})$. This function is

$$v/E_{tot} = \frac{(k_1k_2k_3S - k_{-1}k_{-2}k_{-3}P)}{k_{-1}k_{-2} + k_2k_3 + k_{-1}k_3 + k_1S(k_2 + k_{-2} + k_3) + k_{-3}P(k_{-1} + k_2 + k_{-2})} \tag{2}$$

This equation is valid at all concentrations of substrate and product. However, it is constrained by an expression relating the microscopic rate constants to the equilibrium constant for the reaction

$$K_{eq} = k_1k_2k_3/k_{-1}k_{-2}k_{-3}. \tag{3}$$

In the original Albery-Knowles derivation, an "efficiency function," defined by eqn (4) was optimized. This efficiency function is related to the inverse of eqn (2). Equations (2) and (4) are not identical—(4) is lacking one term. As the missing term contains only K_{eq}, S, and P as components, derivatives taken with respect to microscopic rate constants are the same, regardless of whether one starts with eqn (2) or (4). However, certain conclusions that may be drawn from eqn (4) directly are incorrect with respect to their dependence on S, P and K_{eq}.

$$E_{tot}/v = \frac{1}{S}\frac{1}{k_1} + \frac{k_{-1}}{k_1k_2} + \frac{k_{-1}k_{-2}}{k_1k_2k_3} + \frac{1}{k_2} + \frac{k_{-2}}{k_2k_3} + \frac{1}{k_3} + \frac{P}{SK_{eq}} + \frac{1}{k_{-2}} + \frac{k_2}{k_{-1}k_{-2}} + \frac{1}{k_{-1}}. \tag{4}$$

In principle, optimal values for the microscopic rate constants can be obtained from eqn (2) simply by differentiation of the equation with respect to the five kinetic parameters that remain after the equilibrium assumption is exploited to remove one variable. The five derivatives, set to zero, yield five equations that can be solved simultaneously to provide optimal values of each rate constant. However, this optimum is both chemically unreasonable and mathematically trivial; the forward rate constants are infinite. Thus, the model must be improved by finding mathematical constraints on eqn (2) that are evolutionarily reasonable and, one hopes, also force the model to give optima that are chemically reasonable.

Two constraints were suggested by Albery & Knowles. The second order rate constants k_1 and k_{-3} are assumed to correspond to the second order rate constants for the diffusion of substrate to enzyme. As rates of diffusion in solution presumably cannot be altered by the structure of the protein, k_1 and k_{-3} are treated as constants in the optimization of eqn (2). As a further approximation, they can be assumed to be equal to k_d, the second order diffusion rate constant.

Even with k_1 and k_{-3} constrained, eqn (2) gives a trivial optimum where k_2 is infinitely large, and a constraint on k_2 is necessary. Albery & Knowles constrained this variable by inroducing a linear free energy assumption (1), where k_2 and k_{-2} are related to the equilibrium constant K_2 by eqn (1) and the following equation

$$k_{-2} = CK_2^{\beta-1}. \tag{5}$$

Using substitutions appropriate to these assumptions, eqn (2) is transformed into an equation of two variables, k_{-1} and K_2, with two microscopic rate constants that are presumed to be constant (k_1, k_{-3}), one (k_3) which is eliminated using the equilibrium assumption, and three other parameters, K_{eq} (invariant for a given reaction), β, and C.

$$V = \frac{(E_{tot}k_{-1}CK_2^{\beta-1}k_{-3}(K_{eq}S - P)}{CK_2^{\beta-1}(k_{-1} + k_1S + k_{-3}P + K_{eq}k_{-1}k_{-3}/k_1) + k_{-1}k_{-3}P + CK_2^{\beta}(k_1S + k_{-3}P) + K_2^{-1}k_{-1}K_{eq}k_{-3}(k_{-1}/k_1 + S)}. \tag{6}$$

If β and C are assumed to be evolutionary invariant characteristics of the reaction being catalysed (as is K_{eq}), this equation in two variables (k_{-1} and K_2) can be solved to give a non-trivial optimum. It is this optimum that defines the kinetically optimal enzyme and that is examined below.

The analytical expression for the optimum is rather complicated, and does not provide an intuitive understanding of how the catalytic efficiency of an enzyme is dependent on variation of the internal equilibrium constant. However, if the partial derivative of eqn (6) with respect to k_{-1} is taken, and set to zero, a simple expression relating k_{-1} to K_2 is obtained

$$k_{-1}^2 = k_dk_2(k_2 + k_{-2})(S + P)/(k_{-2}K_{eq}) \tag{7}$$

where $k_d = k_1 = k_{-3}$.

The derivative set equal to zero in eqn (7) was given the name the "uniform binding constraint" (Albery & Knowles, 1976). Some derivations that examine this derivative first justify this in terms of a particular model of enzymic evolution. In

this model, it is assumed that mutations that affect the free energies of bound states uniformly are more common than mutations that influence the energies of bound states differently. While this model may be arguable (Li *et al.*, 1983), it is not a necessary assumption to exploit the mathematical model to find optimal values of K_2 and k_{-1}. The optimum found by setting partial derivatives to zero is a global one. It does not depend on the order in which the derivatives are taken.

When eqn (7) is substituted into eqn (6), it yields an equation for the velocity of the enzymatic reaction as a function of only K_2, the internal equilibrium constant

$$v/E_{\text{tot}} = \frac{JCK_2^{(\beta-0.5)}}{\{LCK_2^{(\beta-0.5)} + MK_2^{0.5} + N[CK_2^{\beta} + CK_2^{(\beta-1)}]^{0.5} + QK_2^{-0.5}\}}$$

where

$$J = k_d^{1.5}[(S+P)/K_{\text{eq}}]^{0.5}(K_{\text{eq}}S - P)$$
$$L = [k_d(S+P)/K_{\text{eq}}]^{0.5}(1+K_{\text{eq}})$$
$$M = [k_d(S+P)/K_{\text{eq}}]^{0.5}k_dP$$
$$N = 2k_d(S+P)$$
$$Q = [k_d(S+P)/K_{\text{eq}}]^{0.5}K_{\text{eq}}k_dS.$$

These are all constant terms, given the assumptions above.

Equation (8) is readily simulated by computer, and these simulations are described below to show how the velocity of an enzymatic reaction is influenced by variations in the internal equilibrium constant K_2, and by variations in the other kinetic parameters that are presumed to be evolutionarily constant for a particular enzyme. These simulations were done on an IBM AT computer.

Equation (8) is derived for an enzyme catalyzing the conversion of one substrate to one product. For enzymes having more substrates or products, the same two contraints (holding rate constants for the binding of substrates and products invariant, and relating rate constants for each chemical step to equilibrium constants for that step by a linear free energy relationship) yield rate equations that give a non-trivial optimum upon differentiation with respect to the remaining kinetic variables. Again, the set of simultaneous equations obtained from this differentiation generally resist analytical solution to give optimal values of the microscopic rate constants. However, these values can be obtained by computer simulation of the equations obtained from the partial differentials.

Consider for example enzymes catalyzing reactions with two substrates and two products that bind in defined order (an ordered Bi-Bi mechanism) (Cleland, 1963)

$$E + A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAB \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EPQ \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EP + Q \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + P + Q. \tag{8}$$

(Here, k_1, k_2, k_{-4}, and k_{-5} are presumed to reflect the diffusion limit, and k_3, and k_{-3} are related to K_3 by a linear free energy relationship. Then, k_5 is expressed in terms of k_{-1} using the external equilibrium assumption, and the partial derivative of the rate equation is taken with respect to k_{-1} and set equal to zero. Optimal

values of k_{-1} (and k_5) are then found by computer simulation. These values are used in the next simulation, where k_4 is expressed in terms of k_{-2} using the external equilibrium assumption, and the partial derivative of the rate equation is taken with respect to k_{-2} and set equal to zero. Optimal values of k_{-2} (and k_4) are then found by computer simulation. This process is iterated until self-consistent values are obtained.

As we shall see, the equations derived above show that the optimal internal equilibrium constant for a catalytically optimized enzyme differ significantly (and predictably) from unity in many cases. Some comment is necessary, to prevent confusion, to show that published derivations of the hypothesis of matched internal thermodynamics, as well as published critiques of the hypothesis, are either incomplete or not valid.

The first proposed derivation of the hypothesis (Albery & Knowles, 1976) was based on the derivation of an expression for K_2 where

$$K_2 = \beta/(1-\beta). \tag{9}$$

Albery & Knowles argued that proving this relationship (eqn (9)) is equivalent to proving that $K_2 = 1$. In this argument, β is assumed to be approximately 0·5 for most reactions; therefore, by eqn 9, $K_2 = 1$. We have previously noted that an assumption that β is approximately 0·5 is tantamount (by the Hammond postulate) to an assumption that $K_2 = 1$ (Nambiar *et al.*, 1983). Therefore, a deduction that K_2 is unity based on an assumption of a β of 0·5 has elements of circularity. In fact, values of β can (normally) be between 0 and 1. Therefore, values of K_2 allowed by eqn (9) can be between 0 and infinity. Further comment on this point can be found in Nambiar *et al.* (1983).

Algebraic inconsistencies within the Albery-Knowles derivation were noted by Chin (1983), who argued that the hypothesis is not valid under "irreversible conditions," but that it remains valid under "reversible conditions". However, this argument in support of this more limited hypothesis is incomplete. In the "reversible" case, Chin sets substrate and product concentrations to their equilibrium values, conditions under which there is no net flux and hence no reaction velocity to optimize. Further, when Chin derives the value of K_2 that gives the optimal flux under equilibrium conditions he finds $K_2 = \beta/(1-\beta)$ (eqn (9)). Again, deriving eqn (9) is incorrectly assumed to be equivalent to proving the relationship $K_2 = 1$.

In an important paper discussing electron transfer enzymes, Rees (1985) attempted a defense of the hypothesis under both reversible and irreversible conditions in response to Chin's critique. This was done by treating β as an evolutionary variable, and finding the values for K_2 that give first derivatives of O with respect to variations in β. Although a solution that gives a stationary point on the surface is found by Rees where $K_2 = 1$, this solution is not in fact an optimum. Rather, the stationary point is a saddle point, where the velocity of an enzyme is maximized with respect to one of the variables but is a minimum with respect to another. This has been independently shown by Rees (personal communication).

Thus, there is no complete algebraic argument in the literature supporting the hypothesis of matched internal thermodynamics for either the reversible or the

irreversible case. However, a convincing critique of the hypothesis has also not been presented.

Chin provides an alternative description for an optimal enzyme operating "irreversibly" which supports our idea of a descending staircase of internal states. However, his derivation formally applies only to the limited case where $P = 0$. Thus, it does not predict, for example, the kinetic parameters of an enzyme that catalyzes a uni-directional flux but may still be subject to product inhibition.

Finally, Raines (1986) has recently presented a discussion based on a mathematically complete efficiency function, and concluded that the optimal internal equilibrium constant lies within certain bounds.

Results

Equation (8) describes a multidimensional surface relating the velocity of an enzyme in terms of a single evolutionary variable, K_2, and a set of parameters. The nature of this surface is of interest as we search for values of the parameters and rate constants that characterize a kinetically optimized enzyme.

Figure 1 shows cross sections of this surface, where the natural logarithm of the velocity is plotted as a function of K_2 for several values of the parameter C (eqn (1)). Values of other parameters are listed in the caption. The physiological conditions are fixed with $P/S = 0{\cdot}01\ K_{eq}$. Thus, the sections of the surface represented in Fig. 1 correspond to an enzyme evolving under physiological conditions where the concentrations of S and P are far from their equilibrium values.

Varying C has the effect of changing the extent to which the chemical step is rate limiting. The bottom trace shows the surface in the extreme where the chemical step is virtually completely rate limiting (C is small).

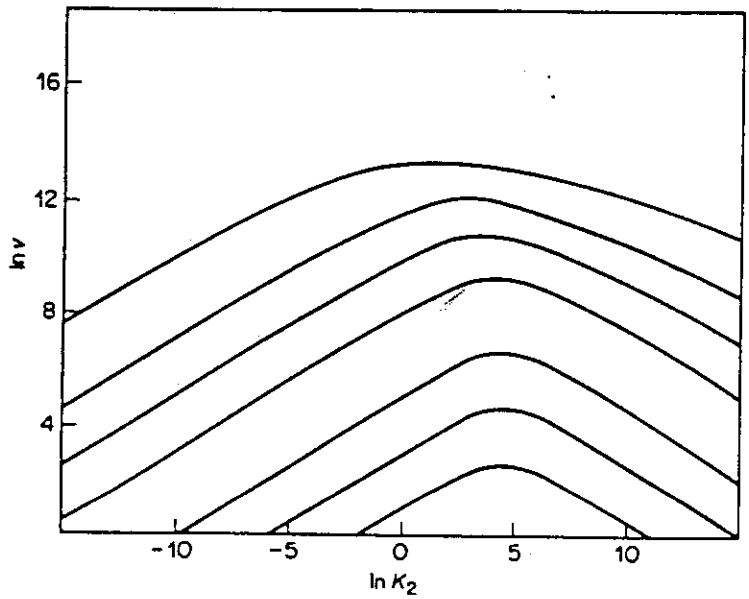

FIG. 1. A plot of $\ln(v)$ versus $\ln(K_2)$, for different values of C, the intrinsic rate parameter for the reaction ES going to EP. $S = 10$ mM, $P = 1$ mM, $K_{eq} = 10$, $\beta = 0{\cdot}5$, $k_1 = k_{-3} = 10^8\ M^{-1}\ sec^{-1}$. Optimal values of K_2 (with the corresponding values of C) are 99 ($C = 1$); 95 ($C = 3$); 88 ($C = 5$); 62 ($C = 8$); 34 ($C = 10$); 13·8 ($C = 12$); 3·5 ($C = 15$).

Two conclusions are apparent from inspection of Fig. 1, both inconsistent with a general notion of matched internal thermodynamics. First, it is apparent from the plot that the optimal internal equilibrium constants under the conditions described in the caption are not unity. The optimal values for K_2 given different values of C are listed in the caption.

Second, if the chemical step remains rate limiting, the optimal value of K_2 is essentially independent of the intrinsic rate parameter C of the linear free energy relationship (eqn (1)). However, as other steps become partially rate limiting, the optimum value of K_2 shifts towards unity, and the optimum broadens.

This is not unexpected. In this model, changing K_2 has two effects, one positive and one negative. First, increasing K_2 increases the flux through the enzyme by making k_2 faster. Second, increasing K_2 decreases the flux through the enzyme by increasing the concentration of the EP complex for any arbitrary value of $[P]$. Figure 1 illustrates the consequence of a tradeoff between these two consequences of varying K_2. As the catalytic step (represented by k_2) becomes less rate limiting, less advantage is gained from an increase in k_2 arising from an increase in K_2, given the concomitant increase in $[EP]$ under steady state conditions.

This result may be a partial explanation for the prevalence of enzymes having an internal equilibrium constant close to unity. As the chemical step in an enzymatic reaction may frequently only be partially rate limiting (Cleland, 1975), equilibrium constants reasonably close to unity are expected to be frequent.

Inspection of Fig. 1 shows that the optimum appears to be rather broad (in this logarithmic plot) as the chemical step becomes less rate limiting. Given this, it is reasonable to ask whether there is any meaningful optimum under conditions where the chemical step is only 10–15% of a rate limiting step for product dissociation, a situation presumed to occur in several classes of enzymes (e.g. dehydrogenases). Figure 2 shows a plot of v versus log K_2 under conditions where the chemical step is only 15% of the product release step. As is evident from the plot, a clear optimum is seen for the internal equilibrium constant ($K_2 = 1{\cdot}15$).

Figure 3 shows how the surface varies as k_1, the rate constant for the binding of substrate, varies. Similar to the results shown in Fig. 1, as k_1 becomes slower, the optimum broadens and the optimal internal equilibrium constant shifts towards unity. While k_1 is normally treated as evolutionarily invariant, this simulation is relevant to the discussion of recent suggestions that substrate reaches the active site by direct transfer from another enzyme (*vide infra*).

Another feature of the surface described by eqn (8) is that the optimal internal equilibrium constant K_2 depends on how far the reaction is from equilibrium under the conditions for which the enzyme is adapted. Figure 4 shows cross sections of the surface for several values for the ratio of concentrations of free substrate and product (S/P) at constant K_{eq}. Values for other parameters are listed in the caption. The optimal K_2 drops from 457 to 1·7 as the P/S ratio varies from 0·001 K_{eq} to 0·5 K_{eq}. As P/S approaches K_{eq}, the internal equilibrium constant K_2 approaches unity. However, contrasting with the matched internal thermodynamics model, an internal equilibrium constant near unity occurs only when the enzyme is catalyzing a reaction very near to its equilibrium.

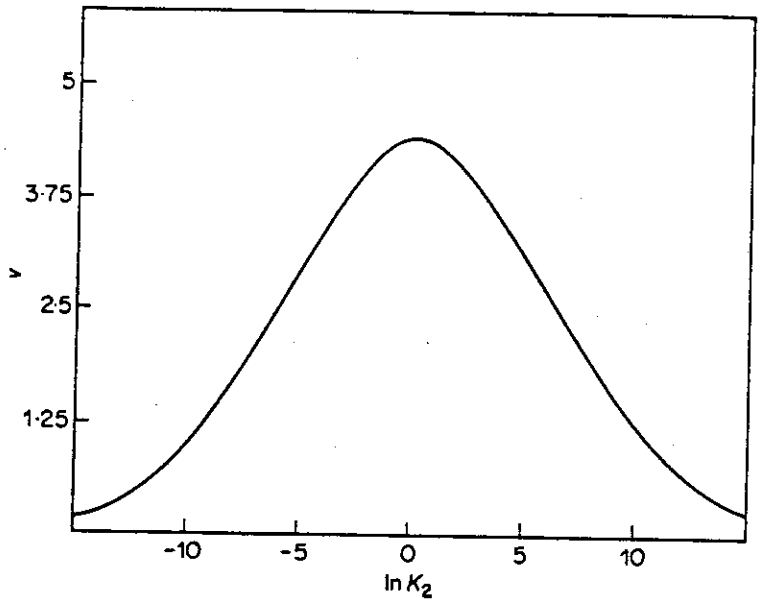

FIG. 2. A plot of reaction velocity versus $\ln(K_2)$ under conditions where the rate constant for the chemical step is approximately 15% of the rate constant for product dissociation. $K_{eq} = 10^{-4}$, $S = 1$ mM, $P = 40$ nM, $k_1 = k_{-3} = 10^8$ M^{-1} sec^{-1}, $\beta = 0{\cdot}5$, $C = 6{\cdot}5$. At the optimum, the value of the rate constant for the release of product is approximately 100/sec, while the rate constant for the chemical step is approximately 700/sec. Nevertheless, the model yields a clearly optimal value of K_2 (1·15).

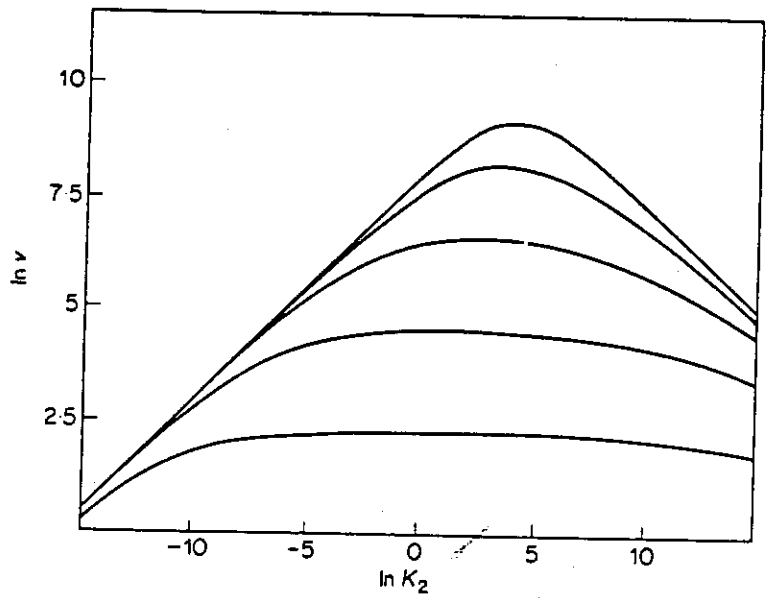

FIG. 3. A plot of $\ln(v)$ versus $\ln(K_2)$ for different values of k_1, the rate constant for binding of substrate. $S = 10$ mM, $P = 1$ mM, $K_{eq} = 10$, $\beta = 0{\cdot}5$, $k_{-3} = 10^8$ M^{-1} sec^{-1}, and $C = 10$. From the bottom to the top, the traces are for values of k_1 that are, respectively: 10^3, 10^4, 10^5, 10^6, and 10^8 M^{-1} sec^{-1}.

Figure 5 shows that the optimal value of K_2 is a function of the parameter β of the linear free energy relationship (eqn (1)). As β varies from 0·3 to 0·7, the optimal K_2 varies from 125 to 28 for a system far from equilibrium ($P/S = 0{\cdot}01\ K_{eq}$). This result is quite consistent with intuition. The larger the value of β, the more k_2 is increased by a given increase in K_2. What is surprising is that for large values of

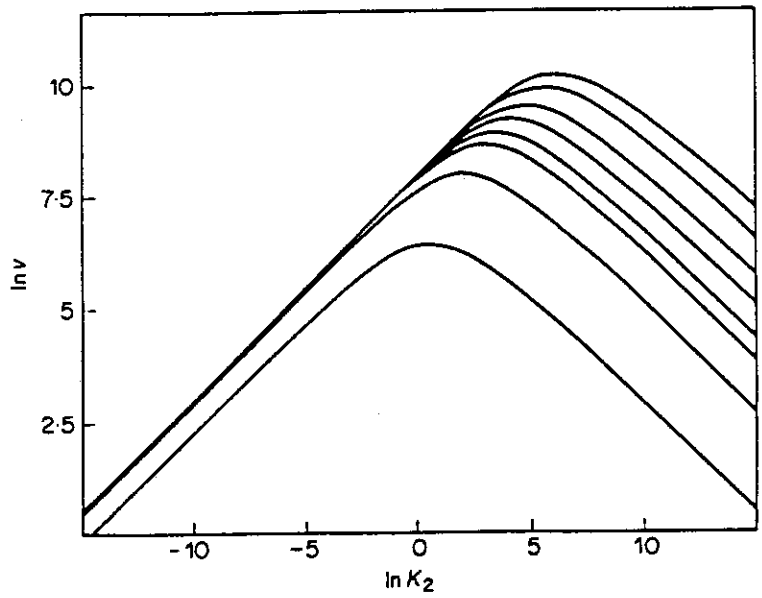

FIG. 4. A plot of $\ln(v)$ versus $\ln(K_2)$, for different values of the ratio of P/S at a constant equilibrium constant (i.e., for changes in the chemical potential driving the reaction). $K_{eq} = 10$, $\beta = 0{\cdot}5$, $k_1 = k_{-3} = 10^8\ \text{M}^{-1}\ \text{sec}^{-1}$, $C = 8$. P is held constant at 1 mM, and S is varied. From bottom to top, optimal values of K_2 (with the corresponding values of S in parentheses) are: 1·7 (0·2 mM), 7·2 (1 mM), 20 (3 mM), 32(5 mM), 62 (10 mM), 113 (20 mM), 251 (50 mM) and 457 (100 mM).

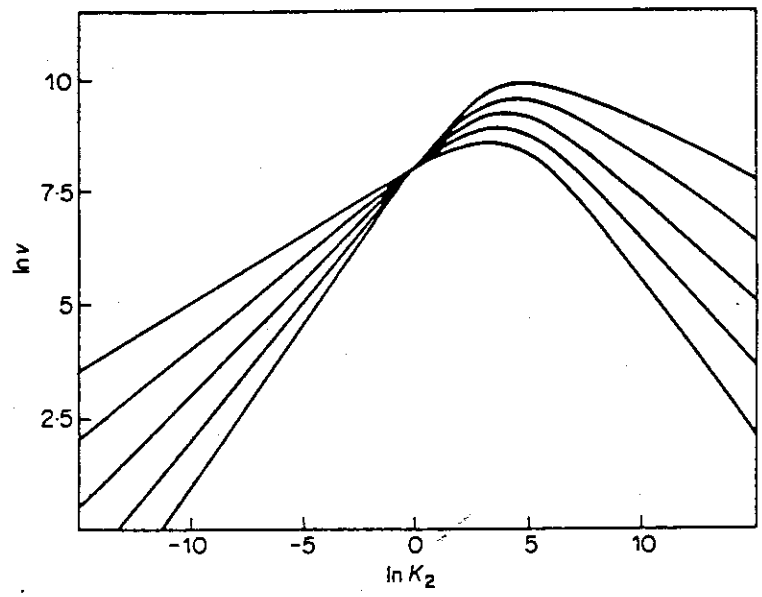

FIG. 5. A plot of $\ln(v)$ versus $\ln(K_2)$, for different values of β, the parameter representing the sensitivity of the rate of the chemical step to the equilibrium constant for the chemical step. Parameters are chosen so that the chemical step is clearly rate-limiting. $S = 10$ mM, $P = 1$ mM, $K_{eq} = 10$, $k_1 = k_{-3} = 10^8\ \text{M}^{-1}\ \text{sec}^{-1}$, $C = 8$. From bottom to top, optimal values of K_2 (with the corresponding values of β) are: 28($\beta = 0{\cdot}3$), 44($\beta = 0{\cdot}4$), 62 ($\beta = 0{\cdot}5$), 88 ($\beta = 0{\cdot}6$), 125 ($\beta = 0{\cdot}7$).

β, where varying K_2 has the most substantial impact on k_2, the internal equilibrium constant for the optimal enzyme exceeds $K_{eq}(S/P)$ if k_2 is rate limiting. For example, when $\beta = 0{\cdot}7$, the optimal internal equilibrium constant is 125, while $K_{eq}(S/P) = 100$.

Discussion

In this model, the complete velocity equation describing the rate of an enzymatic reaction is constrained by making the following assumptions, both following the analysis of Albery & Knowles (1976):

(a) The second order rate constants describing the rate of binding of S and P are not subject to evolutionary variation.

(b) The rate of the chemical step converting ES to EP is constrained by a linear free energy relationship relating the rate to the equilibrium constants of the reaction.

Assumption (b) is consistent with data from Fersht's laboratory, which suggest that there exists a class of mutations where perturbations in kinetic behavior appear to be related to perturbations in internal thermodynamic behavior by a linear free energy relationship (Fersht *et al.*, 1986). Assumption (a) may not be true for all enzymes (*vide infra*), but it may be a serviceable approximation for many (Cleland, 1975).

These two constraints are sufficient to permit mathematical optimization of the complete rate equation to give reasonable values for k_{-1} and the internal equilibrium constant K_2 for a kinetically optimal enzyme.

The model also makes two qualitative predictions concerning K_2. The first, agreeing with the picture from the "descending staircase" model, is that the internal drop in free energy going from the ES complex to the EP complex will be in the same direction as the external free energy drop under the physiological conditions for the enzyme. Further, to the extent that the catalytic step is only partially rate limiting, internal equilibrium constants will tend towards unity.

Both of these qualitative predictions are generally consistent with experimental data. For example, the experimental fact that K_2 for many enzymes is close to unity may be explained as a consequence of two well known facts: (a) for many enzymes, the catalytic step is not fully rate limiting; and (b) many enzymes operate physiologically under conditions where the substrates and products are close to equilibrium. Conversely, enzymes catalyzing reactions far from equilibrium (e.g., fructose bisphosphatase), and enzymes having chemical steps that are rate limiting (e.g. pyruvate kinase) are expected to have internal equilibrium constants farther from unity, with the internal equilibrium constant favoring the enzyme–product complex.

Further, the model makes an interesting qualitative prediction in the case of isozymes that have evolved to catalyze the same reaction in opposite directions. The model qualitatively predicts that the internal equilibrium constant will be a descending staircase downhill in the direction of the flux, regardless of which step is rate limiting, and regardless of the values of various parameters.

This prediction is also consistent with available experimental data. For example, Kaplan (1968) suggested that the two isozymes of lactate dehydrogenase, one from muscle and one from heart, have evolved to catalyze the same reaction in opposite

directions. Lactate dehydrogenase from muscle catalyzes the conversion of pyruvate to lactate; lactate dehydrogenase from heart catalyzes the conversion of lactate to pyruvate. Consistent with a qualitative expectation based on the model, the lactate dehydrogenase from muscle is reported to have an internal equilibrium constant of approximately 4 favoring the enzyme-lactate-NAD^+ ternary complex (Gutfreund, 1975). In contrast, the lactate dehydrogenase from heart is reported to have an internal equilibrium constant for lactate dehydrogenase from approximately 3 in favor of the enzyme-pyruvate-NADH ternary complex (Schwert *et al.*, 1967). While there remain reservations regarding the ability of experimental methods used in these studies to unambiguously measure the relevant internal equilibrium constants, reexamination of these results using different methods has produced the same qualitative results (Nambiar *et al.*, 1983; Ellington, unpublished).

The isozymes of lactate dehydrogenase have been sequenced; they are clearly homologous (Eventoff *et al.*, 1977). The divergent internal equilibrium constants therefore reflect either non-functional drift, or functional adaptation in two lactate dehydrogenases optimized for two different environments. The magnitudes of the divergent internal equilibrium constants are qualitatively consistent with expectations based on the functional model presented here. While more measurements of K_2's for pairs of isozymes are necessary to make a convincing case, this one confirming instance suggests that the divergence of K_2 in lactate dehydrogenases is functionally adaptive.

At a semi-qualitative level, the model may also make predictions, subject to certain limitations. For example, hexokinase and fructose bisphosphatase both catalyze phosphoryl transfer reactions. Hexokinase catalyzes the transfer of a phosphoryl group from a phosphate to an alcohol. The reaction is exergonic; the β value is expected to be small from the Hammond postulate. In contrast, fructose bisphosphatase catalyzes the transfer of a phosphoryl group from an alcohol to water. Neglecting the ionization of the product (which presumably occurs in a subsequent step of the reaction, not in the catalytic step), the reaction is approximately isoenergetic. The β may be near 0·5.

Under physiological conditions, the reaction catalyzed by both enzymes is rather far from equilibrium. Further, in both enzymes, the chemical step is apparently not rate limiting (Rahil *et al.*, 1982; Wilkinson & Rose, 1979). Thus, semiqualitatively, the model suggests the expectation that the internal equilibrium constants for fructose bisphosphatase would be larger than for hexokinase. Semi-qualitatively, this is what is reported. (Rahil *et al.*, 1982; Wilkinson & Rose, 1979).

Quantitative predictions are similarly limited by the need to estimate values for individual parameters in the model. Two limitations are especially serious.

First, to make quantitative predictions, a value for β is needed. Values of β for some chemical reactions can be estimated from the literature, and might be used in conjunction with the model to make quantitative predictions. Of course, the relevant β should relate the rate of the reaction to the equilibrium constant for the reaction (as opposed to relating the rate of the reaction to the pKa of one of the reactants).

However, the relevance of a β value for a reaction in solution to an analogous reaction in an enzymic active site is uncertain. Formally, the β in the model above refers to the change in rate as a function of change in equilibrium constant for those mutations that affect energies of bound species linearly across a reaction coordinate. However, the position of a transition state in the enzymatic reaction need not be the same as the reaction in solution; thus, the β in solution need not be the β for an entirely analogous reaction in the active site.

Only partial solutions to this problem can be offered at this time. For reactions where the catalytic step is not fully rate limiting, the predicted values for K_2 are not strongly dependent on β. In those cases where the catalytic step is rate limiting. β must be guessed from solution reactions and the Hammond postulate, with an understanding of how an error in β would influence the predicted values of the internal equilibrium constant. Finally, molecular biological techniques now permit replacement of individual amino acids in a protein, and the measurements of rate and equilibrium constants in the altered proteins. Thus, the β for an enzymatic reaction is at least in principle experimentally accessible. The recent elegant work of Fersht and co-workers (1986) shows how these methods might be used to determine a β for a specific enzyme.

Regarding the second limitation, in the model, C is treated as a parameter that is evolutionary constant. To use the model to make quantitative predictions, a value for C is needed. This value can be estimated from experimental data, either from an absolute rate constant for the chemical step, or a ratio of rate constants for the chemical and the rate limiting step. An internal equilibrium constant (K_2) is then calculated that is both optimal (as defined by the model) and gives microscopic rate constants that fit these experimental values.

To illustrate the use of this model for semi-quantitative estimation of the internal equilibrium constant (and the limitations of the model as well), an estimate of the internal equilibrium constant was made for the enzyme pyruvate kinase. Physiologically, pyruvate kinase operates far from equilibrium to form ATP and pyruvate from ADP and phosphoenolpyruvate (PEP). Approximate values for the physiological concentrations of substrates and products are known (Srivastava & Bernhard, 1985). The rate limiting step for the enzymatic reaction is probably the interconversion of the ternary complex (Reynard *et al.*, 1961). Therefore, a value of C was chosen to make the chemical step clearly rate limiting.

A value of 0·2 was chosen for β. Skoog & Jencks (1983) report a value of $\beta = 0{\cdot}17$ for an analogous phosphoryl transfer reaction between pyridines. Alternatively, for the enzyme-catalyzed attack of pyrophosphate on a phosphomonoester, Fersht and co-workers (1986) report a value of $\beta = 0{\cdot}21$.

Using these values, and a kinetic scheme that involves rapid binding of substrates and products (Reynard *et al*, 1961), the model yields a calculated value of 16 for the internal equilibrium constant of pyruvate kinase. This is similar to a value measured experimentally (10–15) (Stackhouse *et al.*, 1985). The sensitivity of the calculated value to changes in the other parameters is shown in Table 1.

In summary, the model presented here can make experimentally testable predictions regarding the direction and approximate magnitude of internal equilibrium

TABLE 1

Determination of the optimal internal equilibrium constant for pyruvate kinase operating under in vivo *substrate concentrations. Values for* K_{int} *were determined by assuming a random bi-bi mechanism with a rapid equilibrium assumption for one substrate/product pair, in this case ADP/ATP. Such an assumption is justified considering the relative values of the different dissociation constants* (*Mildvan* et al.). *The rate equation for such a mechanism is identical in form to that for a uni-uni reaction and hence was subject to simulation in a manner similar to that used above. The value of C was chosen so as to make the catalytic step rate limiting for the reaction* (*Boyer* et al.). *The values for parameters not explicitly varied are:* $k_{PEP,on} = k_{PYR,on} = k_{ADP,on} = k_{ATP,on} = 1 \times 10^8$; $k_{ATP,off} = k_{ADP,off} = 1 \times 10^4$ (*Mildvan* et al.); $K_{eq} = 3333$ (*in direction of pyruvate formation*) (*Srivastava & Bernhard*, 1986); $ATP = 8030\ \mu M$ (*Srivastava & Bernhard*, 1986); $K_{eq} \times PEP \times ADP/PYR \times ATP = 65$. *As expected, the optimum is sensitive to the ratio of the substrate and product concentrations and to the choice of a value for* β. *As the catalytic step becomes less rate limiting, the optimum shifts towards one, as seen in Figs* 1–3

PEP(μM)	PYR(μM)	β	C	ADP(μM)	K_{int}
65	380	0·2	0·1	926	16·4
130	380	0·2	0·1	926	33·4
65	760	0·2	0·1	926	8·3
65	380	0·2	0·1	1852	31·6
65	380	0·25	0·1	926	21·9
65	380	0·15	0·1	926	11·5
65	380	0·5	0·1	926	65
65	380	0·2	1·0	926	15·5
65	380	0·2	3·0	926	12·3

constants in enzymes. To the extent that experimental data are consistent with these predictions in an enzyme, the detailed thermodynamic behavior of the enzyme will appear to be functional, reflecting not only the intrinsic free energies of the substrate and product, but also the concentrations of S and P under the physiological conditions for which the enzyme is adapted.

If this suggestion is true, it has a substantial impact on the biologist's view of molecular evolution. Currently, consistent with the "neutral theory of molecular evolution" (Kimura, 1982; King & Jukes, 1969), such detailed behaviors of enzymes would be candidates for "neutral" variation, variation that has no impact on the survival of the host organism and therefore must be non-functional. Careful reexamination of the data and internal equilibrium constants is now necessary to explore this issue.

However, it is equally important to note potential ways in which this model might be criticized.

First, Srivastava & Bernhard (1985) have noted that the concentrations of many enzymes in the glycolytic pathway are substantially higher than the dissociation constants of several of their substrates. Thus, these authors have argued that substrate finds its way into the active site of an enzyme by direct transfer from the enzyme

preceeding it in the metabolic pathway. For enzymes where this is true, k_1 and k_{-3} reflect rates of direct transfer from protein to protein, not the rate of diffusion of small molecules in solution. Therefore, these rates would not necessarily be evolutionarily constant, and assumption (a) must be modified.

Further, for those enzymes where Bernhard's argument applies, the quantitative aspects of the model must be modified. The chemical potentials of S and P become the chemical potentials of S bound to the preceeding enzyme in the pathway, and P bound to the following enzyme. This modification does not appear to affect the qualitative picture of the "descending staircase" model, where only the direction of flux in a metabolic pathway must be known to make predictions. However, data needed for the modified model to make semi-quantitative predictions are generally not available. As we do not yet know for how many enzymes Bernhard's argument is applicable, for now the simpler model seems preferable as a working hypothesis.

Also, almost every new experiment that examines the kinetic details of enzymatic reactions reveals further complexity, especially for enzymes with more than one substrate and product (e.g. Dougherty & Cleland, 1985). In many cases, these complications have little kinetic significance, and do not interfere with comparing predictions from the model with experimental data. However, caution is necessary, as the simple reactions schemes used in our model are probably rigorously accurate for only a very few enzymes. Problems potentially exist in measuring internal equilibrium constants when experimental data collected on an enzyme with a complex kinetic mechanism are interpreted in terms of a simpler kinetic mechanism. These problems have only begun to be explored.

Finally, we must again caution that catalytic optimality as defined in this model may not be the only evolutionary goal, even with regards to the purely catalytic properties of an enzyme (Stackhouse *et al.*, 1985). What is almost certainly biologically relevant is the total flux of substrate to product at the level of the metabolic step, rather than the microscopic flux through a single enzyme molecule. The same flux can be obtained with a fewer enzyme molecules that each are faster catalysts (diffusion limited), or with more enzyme molecules that are each somewhat slower catalysts. A reasonable guess is that the evolutionarily optimal strategy is to create enzymes that produce the desired flux at a minimum metabolic "cost". If diffusion limited enzymes are inherently expensive (perhaps because they are large, or less stable, or require scarce co-factors), one might expect these "cost" constraints to result in the evolution of slower enzymes.

Each of these caveats is a complication that, if taken too seriously, will discourage experimental work. Thus, the model presented here should be regarded as a working hypothesis. Measurement of the detailed thermodynamic behavior of enzymes is a probe into the heart of the catalytic phenomenon. The ability to make predictions should aid significantly in advancing our understanding of enzymes, and how they evolved to their present state.

We are indebted to Professor F. H. Westheimer, Professor D. Arigoni, and Dr Simon Moroney for reading several versions of the manuscript and providing many helpful comments and criticisms. We are also indebted to Professor J. R. Knowles and members of his group for much stimulating commentary.

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