is very similar. The transition states for each of the enolization processes have been stabilized to the same extent.

The evolutionary state of triosephosphate isomerase as a catalyst and the factors leading to the acceleration by the enzyme of the enolization of dihydroxyacetone phosphate by more than 10^9 will be discussed in more detail in the following paper (Albery and Knowles, 1976b).

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Evolution of Enzyme Function and the Development of Catalytic Efficiency†

W. John Albery and Jeremy R. Knowles*

ABSTRACT: An efficiency function is proposed that describes the effectiveness of a catalyst in accelerating a chemical reaction. This function depends on the rate constants for the reaction and has a limiting value of unity when the rate of the reaction is controlled by diffusive steps. The evolution of enzymes toward catalytic perfection can be quantified by this function. For the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde phosphate, the efficiency function has values of 2.5×10^{-11} for a simple carboxylate catalyst and 0.6 for the glycolytic enzyme triosephosphate isomerase. Thus the enzyme is almost a perfect catalyst. The improvement in the catalytic efficiency of enzymes, compared with simple organic molecules, is separated into three broad types of alteration to the Gibbs free-energy profile. In order of increasing difficulty these are "uniform binding," "differential binding," and "catalysis of elementary steps." For changes in "uniform binding," the free energies of the bound states remain the same relative to each other but are altered with respect to those of unbound states. Changes in the "differential binding" are more subtle and require the enzyme to discriminate between different bound intermediates. Finally, the most sophisticated improvement involves "catalysis of an elementary step," where an enzyme must discriminate between the transition state and the ground state of the kinetically significant step. These concepts are discussed for enzyme catalysis generally and are applied in particular to the reaction catalyzed by triosephosphate isomerase.

In the previous paper (Albery and Knowles, 1976b) the Gibbs free-energy profile for the reaction catalyzed by triosephosphate isomerase was derived. Qualitatively, it appears from the profile that this enzyme has reached the end of its evolutionary development. Moreover, a comparison of the rates of enolization of dihydroxyacetone phosphate in the presence and absence of the enzyme (Hall and Knowles, 1975) shows that the enzymic reaction is about 10^9 times faster. In this paper we first develop a quantitative measure of the catalytic effi-

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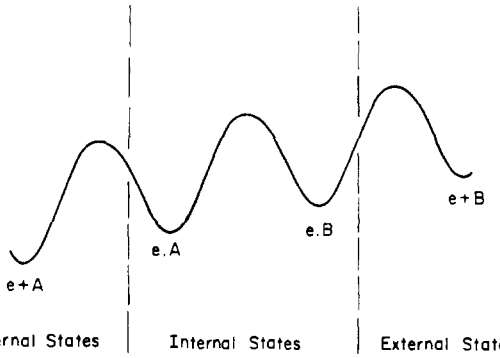

FIGURE 1: The free-energy profile for a simple catalyzed reaction. e is the catalyst and A and B are the substrate and product.

ciency of an enzyme, and then explore how this efficiency function varies with three features of the free-energy profile: the uniform binding of all the reaction intermediates to the enzyme; the differential binding of different intermediates to the enzyme; and the catalysis of the covalency changes of the reaction itself. These general concepts are discussed with particular reference to the energetics of triosephosphate isomerase. The treatment presented is derived for a system where catalyst and substrates diffuse freely in solution, and will be most relevant to enzymes not involved in metabolic control.

The System

Although the concepts developed in this paper are general ones, we shall discuss them with reference to the simplified pathway for an enzyme-catalyzed reaction involving one substrate, one product, and a single step for their interconversion:

$$e + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} e \cdot A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} e \cdot B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} e + B \qquad (1)$$

The substrates A and B have concentrations a and b, and the total concentration of enzyme e is e_0. The Gibbs free-energy profile[1] for such a system will have the form illustrated in Figure 1, and in our discussion, we shall distinguish between the "internal" and "external" states as shown.

The Efficiency Function

The individual enzymes in metabolic pathways may operate under different constraints. For instance, the concentration of the substrate pool may be constant, or the flux through the particular enzyme system may be controlled by the turnover rate of an enzyme higher in the pathway. Furthermore, the particular enzyme system may operate under either "irreversible" or "reversible" conditions as:

"irreversible":

$$e + A \xrightarrow{\text{rate determining}} e + B \xrightarrow{\text{fast}} \text{subsequent products}$$

"reversible":

$$e + A \underset{\text{near equilibrium}}{\rightleftharpoons} e + B \xrightarrow{\text{slow}} \text{subsequent products}$$

In order to define catalytic efficiency, we start from the obvious

[1] While the Gibbs free-energy profiles are calculated on the basis of the standard equation: $\Delta G^{\ddagger} = -RT \ln (kh/k_B T)$, they should not, for the purposes of this paper at least, be viewed as more than convenient illustrative summaries of the experimentally derived rate constants.

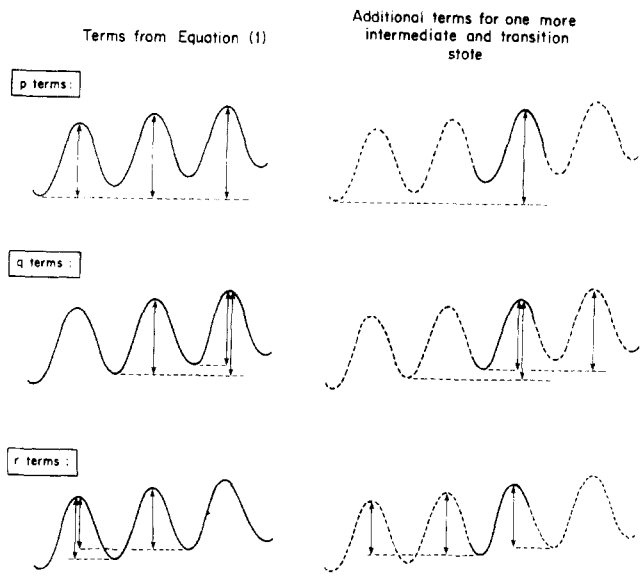

FIGURE 2: Illustration of the free-energy differences represented by the terms of eq 1.

position that an efficient enzyme will mediate a high flux of substrate to product, and for an enzyme working under irreversible conditions we can express this as:

$$y = e_0/v \qquad (2)$$

where e_0 is the enzyme concentration and v is the moles of A converted to B per unit volume and time. For this case, y is the reciprocal of the turnover number of the enzyme. But if the enzyme operates under reversible conditions, the back reaction will be significant, and since it is the *net* flux of A to B that concerns us, y must be modified to take account of how far the system is perturbed from equilibrium. This is achieved by writing:

$$y = e_0(1 - b/K_e \cdot a)/v \qquad (3)$$

where K_e is the overall equilibrium constant for the interconversion of A and B. Equation 3 is general and reduces, of course, to eq 2 when $a \gg b$, i.e., under irreversible conditions.

Using the steady-state approximation, it can be shown that in terms of the rate constants of eq 1:

$$y = p/a + q + \theta r \qquad (4)$$

where:

$$p = \frac{\vec{K}_m}{\vec{k}_{cat}} = (1/k_1 + k_{-1}/k_1k_2 + k_{-1}k_{-2}/k_1k_2k_3) \qquad (5)$$

$$q = 1/\vec{k}_{cat} = (1/k_2 + k_{-2}/k_2k_3 + 1/k_3) \qquad (6)$$

$$r = 1/\overleftarrow{k}_{cat} = (1/k_{-2} + k_2/k_{-2}k_{-1} + 1/k_{-1}) \qquad (7)$$

The value of θ is zero for the "irreversible" case and unity for the "reversible" case. Each term in the expressions of eq 5, 6, and 7 is associated with a single free-energy difference, as illustrated in Figure 2. The expression for y can be extended for a reaction having more internal states, by adding appropriate terms to p, q, and r in accordance with the principles shown in Figure 2.

The form of eq 4 shows that y is a function of all the rate constants, and of the substrate concentration, a. The concentration of A appears in the first term of eq 4 because every term in the expression for p (eq 5) contains the second-order rate

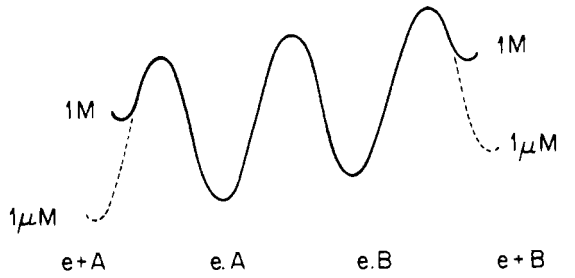

FIGURE 3: The effect of the choice of standard state on the appearance of a free energy profile for the catalyzed interconversion of A and B.

constant, k_1. Since we are ultimately interested in the effectiveness of the enzyme in vivo, we must use the in vivo concentration of A, a°, to define a standard value for y, y° A particular advantage of this definition of y° is that we can use this in vivo substrate concentration as a standard state in the construction of a Gibbs free-energy profile that has to contain both first- and second-order rate constants. As illustrated in Figure 3, the appearance of such free-energy profiles depends on the choice of standard state. The choice of the in vivo concentration means that the profile most clearly illustrates the relative free energies of the species involved. In particular, the relative stabilities of free enzyme and bound enzyme are shown by the positions of the minima for e + A, e·A, and e·B. Whatever the choice of standard state, the e + B minimum (relative to the e + A minimum) represents the overall thermodynamics.

Combining eq 2, 3, and 4, we obtain:

$$y = e_0\left(1 - \frac{\theta b}{K_e a}\right)/v = p/a + q + \theta r \qquad (8)$$

Taking logarithms, differentiation and rearrangement gives.

$$\mathrm{d}(\ln e_0) - \mathrm{d}(\ln v) + \left(\frac{\theta b}{K_e a - \theta b} + \frac{p/a}{p/a + q + \theta r}\right)\mathrm{d}(\ln a) - \frac{\theta b}{K_e a - \theta b}\mathrm{d}(\ln b) = \frac{a^{-1}\,\mathrm{d}p + \mathrm{d}q + \theta\,\mathrm{d}r}{p/a + q + \theta r} \qquad (9)$$

The right-hand side of this expression contains the terms describing changes in the rate constants, while the left-hand side contains the terms describing changes in the concentrations of enzyme, substrate, and product, and changes in the velocity of the reaction. The purpose of eq 9 is to relate the efficiency of catalysis (which is derivable from the rate constants for the reaction) to the four variables (e_0, v, a, and b) that together define how effective a particular enzyme is in a metabolic pathway. The equation as a whole describes the direction in which the four variables on the left-hand side change, with changes in the rate constants for the reaction. *Any change in a rate constant that results in the right-hand side being negative leads to an enzyme of greater catalytic efficiency, whichever of the four variables on the left-hand side alter as a result.* To show this, let us take each variable in turn while holding the other three constant. If an enzyme becomes a more efficient catalyst, then a lower concentration of it can maintain the same flux in the metabolic pathway, and $\mathrm{d}(\ln e_0)$ in eq 9 is negative. The biosynthesis of enzyme protein is expensive in free-energy terms, so an organism that requires less enzyme will clearly have a selective advantage. Secondly, the enzyme will be catalytically more efficient if it can increase the net velocity of the reaction, for the same concentrations of enzyme, substrate, and product (i.e., $\mathrm{d}(\ln v)$ is positive). Thirdly, the

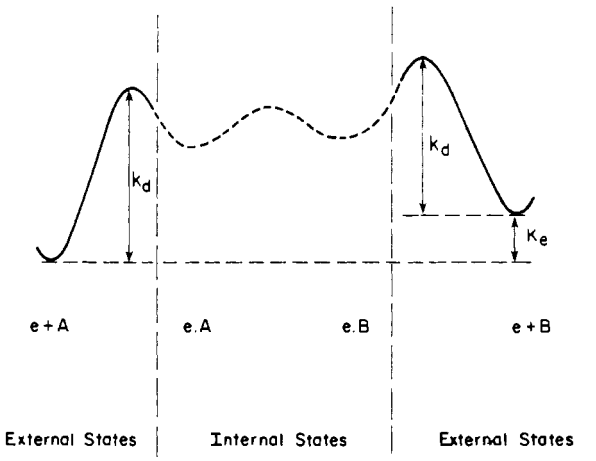

FIGURE 4: The free-energy profile for a perfect catalyst where the catalyst and its substrates diffuse independently. k_d is the rate constant for diffusion of A or B to the catalyst; K_e is the overall equilibrium constant for the reaction.

enzyme will be catalytically more efficient if the same quantity of enzyme can produce the same net velocity from a smaller concentration of substrate (i.e., $\mathrm{d}(\ln a)$ is negative). Finally, the fourth term (in product B) only arises when the enzyme is operating under (or near to) reversible conditions. Again, the enzyme will be catalytically more efficient if it can maintain the same net velocity with a larger quantity of product present (i.e., $\mathrm{d}(\ln b)$ is positive). [In drawing the last two conclusions, it should be noted that in order to maintain a flux in the forward direction, $K_e a$ must always be greater than θb.] An advantage for an enzyme in a metabolic pathway of improved catalytic efficiency leading to a reduction in a and/or an increase in b arises from the fact that A is itself the product of the previous enzyme-catalyzed reaction, and B the substrate for the subsequent one. A lower concentration of A decreases the rate of the back reaction through the previous step or steps; a higher concentration of B increases the forward rate through the subsequent step or steps.

Thus we may expect that natural selection will lead to changes in the rate constants that result in the right-hand side of eq 9 being negative. In terms of the previously defined quantity y°, we can express this conclusion as: $\mathrm{d}y^\circ < 0$. Evolutionary pressure on an enzyme that is uncomplicated by any control function always acts to reduce y°. Just as Gibbs free energy is a measure of the irreversible approach to chemical equilibrium, so y° is a measure of irreversible evolutionary improvement in catalytic efficiency. At chemical equilibrium the free energy is at a minimum, and we may enquire whether y° has a minimum value. In the free-energy profile shown in Figure 2, y° will decrease as the free-energy differences between the maxima and the minima are decreased. While it is possible for the positions of the internal transition states and internal intermediates (see Figure 1) to be brought closer together until the terms they contribute to y° become kinetically insignificant, this is not true of the external states. The overall equilibrium constant of the reaction depends only upon the chemical nature of A and B and cannot be affected by the enzyme. This fixes the relative positions of the external states e + A and e + B. The two external transition states relate to the reactions e + A → e·A and e + B → e·B, and neither of these can be faster than the diffusion limit. Hence the profile and the two terms corresponding to the minimum value of y° are illustrated in Figure 4, and we may write (by inspection of Figure 2 and eq 4):

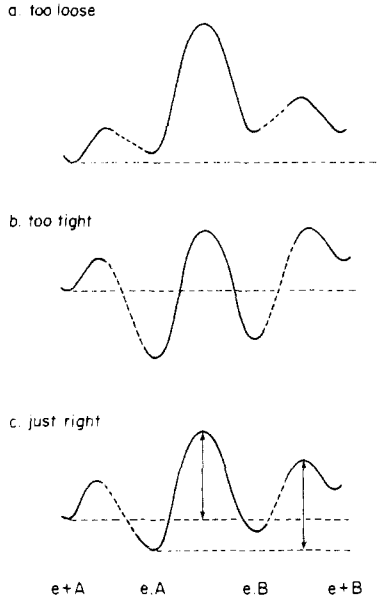

FIGURE 5: The effect of changes in uniform binding of the internal states. The indicated free-energy differences are equal when the uniform binding criterion is satisfied (see the text and eq 12).

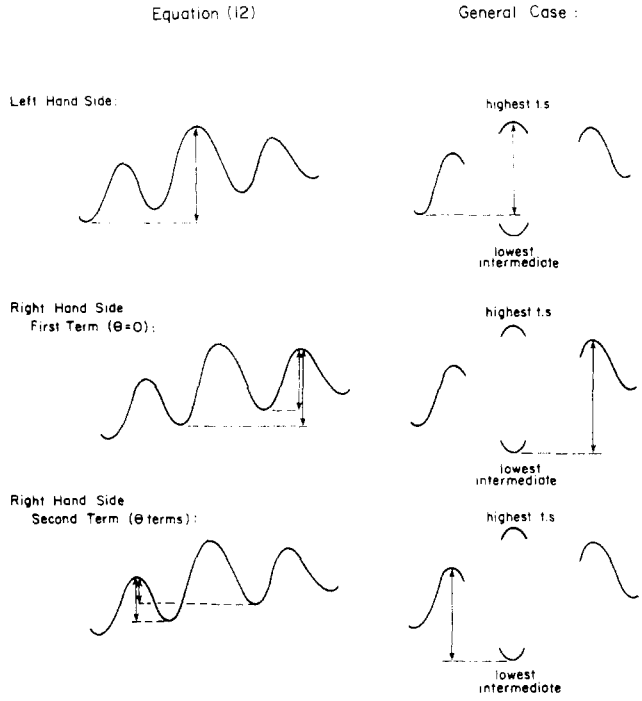

FIGURE 6: Illustration of the free-energy terms of eq 12

$$y_{min}^{\circ} = 1/k_d a^{\circ} + 1/K_e k_d a^{\circ} \qquad (10)$$

where k_d is the rate constant for the diffusion-controlled reaction. Therefore, y°, like the Gibbs free energy, does have a minimum value, which represents the perfect catalyst. We can, therefore, define an "efficiency function", E_f, as:

$$E_f = y_{min}^{\circ}/y^{\circ} \qquad (11)$$

For a perfect catalyst this function equals unity. Since both terms of eq 10 depend on a (unlike eq 8 where some terms are independent of a), this conclusion is unaffected by the value of the ambient substrate concentration which we chose as the standard state. Catalysts that fall short of perfection have, of course, values of E_f less than one, and the value of E_f now may depend on the value of the ambient substrate concentration (see later).

Types of Thermodynamic and Catalytic Improvement

Next, we discuss how different types of change in the free energy profile affect the efficiency function. As discussed above, the positions of the four external states (Figure 1) are fixed and cannot be changed by any evolutionary development of the enzyme. (It must be stressed that the external states in Figure 1 represent simply the diffusion-limited "on" reactions for the binding of each substrate to the enzyme.) We are therefore concerned with the positions of the internal states relative to the framework provided by the external states and suggest three kinds of alteration in the internal states.

Uniform Binding. We propose that the easiest (i.e., most probable) change is one that we may call "uniform binding". In this type of change, *the positions of all the internal states are shifted energetically up or down by the same amount* (relative to the external state framework). The reason why we consider this to be the easiest type of change is that it is nonspecific and affects all substrate (and intermediate) species equally: the enzyme does not have to discriminate amongst the different internal states. Changes in uniform binding may involve parts of the bound substrate remote from the reaction center and may be effected by any mechanism (e.g., electrostatic, H bonding, hydrophobic interaction, etc.). Because of the multiplicity of binding mechanisms and the fact that discrimination amongst substrate species is unnecessary, this type of change may well have evolved most easily (this proposition is discussed more fully later).

In Figure 5, we illustrate the effect of changes in uniform binding. The profile of Figure 5a shows a situation where there is insufficient binding of the internal states and the central transition state presents a large barrier to the reaction. In Figure 5b the binding is much tighter and the central barrier is now kinetically insignificant. However, the problem now becomes the release of enzyme and product from the stable e·A intermediate. Effectively, turnover is reduced because the enzyme is tied up in the e·A complex. The balance between these two inefficient profiles is shown in Figure 5c where the indicated free-energy differences are equal. This equality may be proved by differentiation of the efficiency function with respect to a change in the free energy of all the internal states. One finds that E_f is a maximum when:

$$\frac{k_{-1}}{k_1^{\circ} k_2} = \left(1 + \frac{k_{-2}}{k_2}\right)\frac{1}{k_3} + \frac{\theta}{k_{-1}}\left(1 + \frac{k_2}{k_{-2}}\right) \qquad (12)$$

where $k_1^{\circ} = k_1 a^{\circ}$ The terms in this equation are illustrated in Figure 6. Similar but more complicated expressions can be obtained for systems involving more internal states. In Figure 6 we have also shown the relevant free-energy differences for a profile where both one internal transition state is significantly above the others and one intermediate is significantly below the others. In many cases, one of the free-energy differences will dominate the sums of free energy terms exemplified by the right-hand side of eq 12; this is true of the example shown in Figure 5c. It may also be noted that the terms involving θ are only important for a downhill reversible reaction. If a reversible reaction is uphill, then, although θ equals 1, each of the terms involving θ will be less than its counterpart in the first term on the right-hand side of eq 12. In this case the most efficient situation arises when the free-energy difference between the more stable reactant and the highest transition state is equal to the free-energy difference between the most stable reaction

intermediate and the transition state for product loss from the enzyme. This is what is shown in Figure 5c.

If one knows the free-energy profile for an enzyme-catalyzed reaction, therefore, one can test, through eq 12, how closely the enzyme satisfies the postulate of uniform binding.

Differential Binding. The second type of alteration to the energetics that we consider involves *changes in the relative stabilities of the internal intermediates, and the consequential effects on the internal transition states.* We propose that, compared with uniform binding, changes that discriminate amongst different internal intermediates are harder to achieve (i.e., are less probable) than uniform binding changes, since they must be more specific and the method employed by the enzyme must be more subtle. For instance, a functional group at the enzyme's active site may distinguish through differential hydrogen bonding between two bound intermediates by virtue of a hydroxyl group in a different position in the intermediate. This discrimination, since it is dependent upon changes in substrate structure during the catalyzed reaction, will have to be restricted to the neighborhood of the reaction center.

One can find how the efficiency function is improved by changes in the levels of the internal intermediates if one can suggest how such changes affect the levels of internal transition states. Imagine the situation illustrated in Figure 7a. In this profile, the dotted lines represent kinetically insignificant states, and the efficiency function is determined by the free-energy differences shown, where t.s.$_I$ is the highest transition state, and i_{III} is the lowest intermediate. From the previous discussion, the efficiency will be improved if the internal thermodynamics are altered so that i_{III} is less tightly bound while the i_I–t.s.$_I$ block is more tightly bound. The limit to this change in differential binding comes when i_I becomes the kinetically significant intermediate (Figure 7b). Differential binding between well-separated intermediates (e.g., i_I and i_{III}) will be easier to achieve than when the intermediates are connected (e.g., i_I and i_{II}) since it will surely be easier to discriminate between structures that are structurally more dissimilar. The internal states should alter, therefore, until the kinetically significant transition state and intermediate are connected, and are those that correspond to the largest free energy difference for an elementary step. This is the situation illustrated in Figure 7b. Now, in order to improve the efficiency further by differential binding, we may alter the relative positions of the intermediates i_I and i_{II} (i.e., those on either side of the highest transition state). For instance, in Figure 7b we may lower i_{II}, which is as yet kinetically insignificant. The effect of changing the relative positions of i_I and i_{II} on the position of the transition state t.s.$_I$ can be assumed to follow a linear free-energy relationship of the Bronsted or Hammett type, and it can be shown (see Appendix) that the optimum situation arises when the level of i_I is the same as that of i_{II}. Qualitatively, this means that as one lowers i_{II} down toward i_I, t.s.$_I$ is also lowered, and the overall reaction flux is increased. This improvement continues until i_{II} becomes level with i_I (Figure 7c). Further change of i_{II} in the same direction would then lower the efficiency since i_{II} would then become the most stable intermediate. Analogous arguments may be applied if initially i_{II} is more stable than i_I. In this case i_I moves down and i_{II} moves up, until they become of equal free energy.

The result of the changes in internal states discussed in this section will therefore be that the *kinetically significant transition state will lie between kinetically significant intermediates of equal free energy.*

Catalysis of Elementary Steps. The only way now remaining in which the catalytic efficiency of the system can be improved is to *reduce the free energy of activation of those elementary steps whose intermediates and transition states are kinetically significant.* This type of change requires the most subtle of all mechanisms. The enzyme may discriminate between a transition state and its adjacent intermediates, and bind the transition state more tightly. Alternatively, the enzyme may develop a new catalytic pathway, for instance, by going from monofunctional to bifunctional (e.g., push-pull) catalysis. [Subsumed under this heading is the mechanistic panacea of many enzymologists: the "microenvironment". If we accept that the microenvironment describes the existence of local regions of low (or high) dielectric constant, and the presence of enzyme groups that allow specific "solvation" (i.e., stabilization) of intermediates and/or transition states, then we need no further special characteristic of enzyme catalysis and can properly lump together all those effects that result in the lowering of the activation free energy of an elementary step.] These improvements will clearly be peculiar to the chemistry of the elementary step involved and are not, therefore, amenable to a general treatment. However, as discussed later, analysis of the free-energy profile in terms of the efficiency function allows us to estimate the contribution to catalytic improvement of the three types of change viz: uniform binding, differential binding, and catalysis of elementary steps.

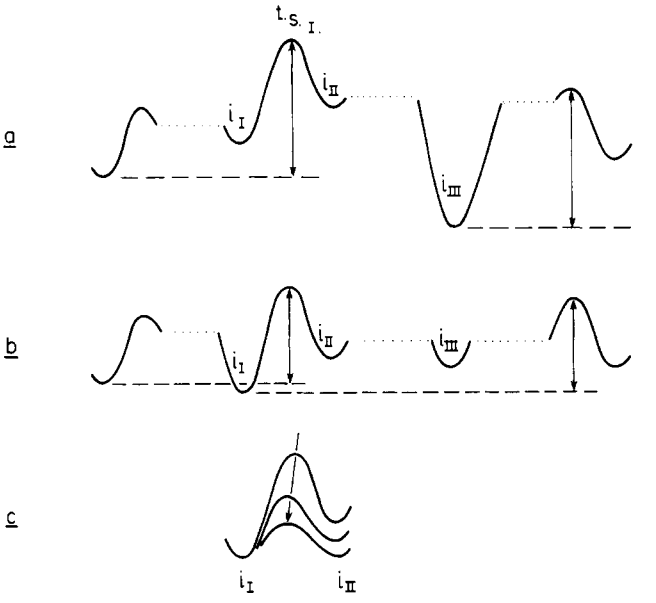

FIGURE 7: Illustration of the effect of differential binding (a) of unconnected intermediates (t.s.$_I$ and i_{III}) followed by uniform binding and (b) of connected intermediates until they are of equal free energy (c).

Application of the Efficiency Function to Triosephosphate Isomerase

We are now in a position to analyze the Gibbs free-energy profile for the reaction catalyzed by triosephosphate isomerase, in terms of the concepts developed above. The profile for this reaction is slightly more complicated (Albery and Knowles, 1976b) than the simplified scheme of eq 1, and we revert to the earlier notation (Albery and Knowles, 1976a):

$$E + S \rightleftharpoons ES \rightleftharpoons EZ \rightleftharpoons EP \rightleftharpoons E + P \qquad (13)$$

where S is dihydroxyacetone phosphate, P is D-glyceraldehyde 3-phosphate, ES and EP are the enzyme-bound complexes of S and P, and EZ is the enzyme-bound enediol intermediate.

From eq 4 to 7, 10 and 11, and the values of the rate constants (Albery and Knowles, 1976b), and using the in vivo value for s° of 40 μM (Williamson, 1965), the value of the efficiency function is:

$$E_f^{\text{isomerase}} = 0.6$$

We can now compare the enzyme catalyst (Genesis, *i*, 20) with the situation before enzymes existed (Genesis, *i*, 10), where the reaction could only be catalyzed by a carboxylate base such as CH_3COO^-. [The reason that we opt for a carboxylate base is to parallel the enzyme, which very probably uses a carboxylate group of glutamic acid 165 analogously (Hartman, 1968, 1970, 1971; de la Mare et al., 1972).] To construct a free-energy profile for the reaction catalyzed by a simple carboxylate, we assume that the base associates with each of the substrates with a dissociation constant of 5 M, at a rate[2] of 3.7×10^8 M^{-1} s^{-1}. We know the rate constants for the enolization reactions of dihydroxyacetone phosphate and of glyceraldehyde phosphate in the absence of enzyme,[3] and the equilibrium percentage of their common enediol (Hall and Knowles, 1975), and we display in Figure 8 the two Gibbs free-energy profiles, for the reaction catalyzed by CH_3COO^-, and for the reaction catalyzed by the enzyme. Taking the same value for the standard state of the substrate of 40 μM, we find that

$$E_f^{\text{acetate}} = 2.5 \times 10^{-11}$$

Hence, compared with a free carboxylate base, the enzyme triosephosphate isomerase is more than 10^{10} times as efficient. Further, since the maximum possible value of E_f is unity, we may conclude that triosephosphate isomerase (E_f, 0.6) is an almost perfect catalyst.[4]

As discussed previously (Albery and Knowles, 1976b), only limiting values for the free-energy levels of the intermediates EZ and EP and of transition state 1 are known. The constraints on these three states are now considered. First, in the present discussion we assume that the step E + S → ES is diffusion controlled with a rate constant of 3.7×10^8 M^{-1} s^{-1} (by analogy with E + P → EP) which makes transition state 1 kinetically insignificant. Secondly, the free-energy level of the state EZ (the enzyme-bound enediol intermediate) can be more closely defined by consideration of the third fate of this species, namely, the rate of exchange of the labile proton between EZ and the solvent (Fisher et al., 1976). The isotope effect on this exchange reaction is near unity, and plausible values for the exchange rate lie in the range of 10^5 to 10^7 s^{-1}. Since the level of the transition state for this exchange is known, this range of values sets reasonable limits on the level of EZ, as is indicated in Figure 8. Thirdly, the free-energy level of EP (the enzyme–glyceraldehyde phosphate complex) can

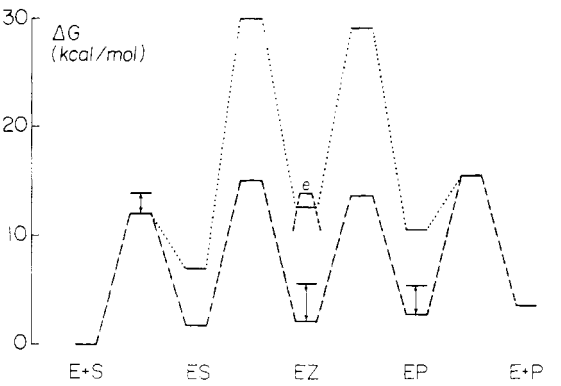

FIGURE 8: Experimental free-energy profiles for the interconversion of dihydroxyacetone phosphate (S) and D-glyceraldehyde 3-phosphate (P). Catalysis either by a free carboxyl group (levels connected by *dotted* lines, rates for CH_3COO^- calculated from Hall and Knowles (1975), according to eq 14) or by triosephosphate isomerase (levels connected by *dashed* lines, from Albery and Knowles (1976b)). The vertical arrows show the limits of those states that are less well defined. The transition state marked e refers to the exchange of protons between the solvent and the enzyme-bound enediol intermediate (EZ).

be considered by reference to the relative free energies of the transition states 2 and 3. In the enzyme-catalyzed reaction, the difference between transition states 2 and 3 is slightly larger than that for the corresponding reaction catalyzed by a simple CH_3COO^- ion (Figure 8, and Hall and Knowles, 1975). Hence we should expect that on going from the reaction catalyzed by CH_3COO^- to the reaction catalyzed by the enzyme, the substrate in EP would be bound at least as tightly as that in ES. This sets an upper limit on EP, indicated in Figure 8 (compare the *lower* limit of EP derived previously, Albery and Knowles, 1976b). Figure 8, therefore, represents the closest approach to the complete free-energy profile for both the enzyme-catalyzed and the carboxylate-catalyzed reactions.

The Development of the Catalytic Efficiency of Triosephosphate Isomerase. On the basis of the efficiency function derived in this paper, and of the free-energy profile summaries (Figure 8) of the rate constants for the isomerization reaction catalyzed by a free carboxylate and by the enzyme, we can discuss the development of catalytic function in terms of uniform binding, differential binding, and catalysis of elementary steps.

First, we apply the argument of eq 12 to the isomerase-catalyzed reaction. The dominant term on the left-hand side of the equation will correspond to the free-energy difference between E + S and transition state 2 (see Figure 6), which we can designate as $\Delta G^{\ddagger}_{\text{LHS}}$. The dominant term on the right-hand side of eq 12 will correspond to the free-energy difference between ES and transition state 4 (see Figure 6), $\Delta G^{\ddagger}_{\text{RHS}}$. These values are $\Delta G^{\ddagger}_{\text{LHS}} = 15$ kcal/mol; and $\Delta G^{\ddagger}_{\text{RHS}} = 14$ kcal/mol. The agreement is reasonable and indicates that the uniform binding criterion has essentially been met.

Next we must inquire how efficient a catalyst would be if (starting with the carboxylate-catalyzed profile of Figure 8) it simply optimized the internal states with respect to the uniform binding criterion. In Figure 9b this profile is drawn: the *relative* free energies of ES through to EP (i.e., all the internal states) are the same as those for the reaction catalyzed by CH_3COO^-. The efficiency function for this profile is:

$$E_f = 3 \times 10^{-6}$$

which indicates that the optimization simply of uniform binding produces a considerable improvement in E_f, of over five orders of magnitude.

[2] This is the "on" rate of D-glyceraldehyde 3-phosphate to triosephosphate isomerase (Albery and Knowles, 1976b).

[3] In previous work (Hall and Knowles, 1975) the rate constants for the reaction have been measured in solutions containing approximately 50 mM HCO_3^-. From these values we can estimate, using the Bronsted relationship, the second-order rate constants for the reaction mediated by a base such as CH_3COO^-, by writing the following equation (which is designated eq 14) $\log k_{CH_3COO^-} = \log (k_{obsd}/0.05) + \frac{1}{2}(pK_{CH_3COOH} - pK_{H_2CO_3})$. [The thermodynamic pK_a of H_2CO_3 of 3.8 (Perrin, 1969) is used.]

[4] The definition of y_{min}° depends, of course, on k_d (see eq 10), and we have argued that the "on" rate of D-glyceraldehyde phosphate (3.7×10^8 M^{-1} s^{-1}) *is* the diffusion limit (Albery and Knowles, 1976b). If the diffusion rate is in fact slightly higher than this, then the value of E_f for the enzyme will be somewhat lower than 0.6, but the arguments will not be significantly affected.

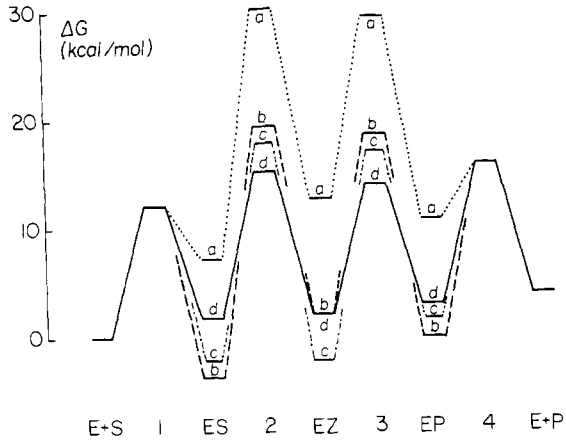

FIGURE 9: Free-energy profiles for different possible catalysts. [a (. . .)] a carboxylate, CH_3COO^-; [b(- - -)] a carboxylate catalyst with optimized uniform binding; [c (·····)] a carboxylate catalyst with optimized uniform binding and optimized internal thermodynamics (note that levels for ES and EZ are here equal); and [d(—)] triosephosphate isomerase. Values of the efficiency functions for these different profiles are reported in Table I.

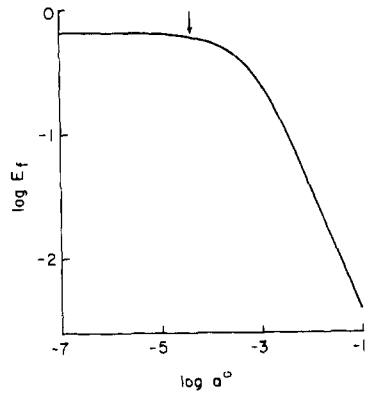

FIGURE 10: Variation of the efficiency function, E_f, for triosephosphate isomerase with a°, the ambient substrate concentration.

TABLE I: Values of the Efficiency Function for Various States of the Catalyzed Interconversion of Dihydroxyacetone Phosphate and Glyceraldehyde Phosphate.

Optimization of	Catalysis by free CH_3COO^-			Catalysis by the Enzyme
Uniform binding	X	√	√	√
Differential binding	X	X	√	√
Catalysis of elementary steps	X	X	X	√
Value of the efficiency function	2.5×10^{-11}	3×10^{-6}	1.5×10^{-4}	0.6

Secondly, we may consider the effect of the optimization of the internal thermodynamics. As shown earlier, the improvement here must result in kinetically significant transition states lying between kinetically significant intermediates of equal free energy, and the application of this principle to the profile in Figure 9c requires the catalyst to equalize the levels of ES and EZ. Although the free energy of EZ is experimentally somewhat uncertain, it seems probable that changes in the predicted direction have taken place, and we find (Figure 8) that the kinetically significant transition state 2 is flanked by the two most kinetically significant intermediates, ES and EZ. It also seems probable that the substrate in EP is bound more tightly than that in ES, which—although EP is itself kinetically insignificant—reduces transition state 3 to kinetic insignificance (see Figure 8).

Once again, we must ask how efficient our putative developing catalyst would be if the internal thermodynamics were optimized, and the appropriate profile (with the levels of ES and EZ equal) is shown in Figure 9c for which

$$E_f = 1.5 \times 10^{-4}$$

This is a significant improvement in efficiency, though because of the fact that for this system the overall equilibrium constant is not very far from unity, there is not much scope for major improvements in efficiency arising from alterations to the internal thermodynamics.

Thirdly, the enzyme may improve its efficiency by the more effective catalysis of the elementary steps. Since the actual enzyme has a value of E_f of 0.6, there is evidently a significant contribution to the catalytic efficiency from the acceleration of the enolization reaction (ES → EZ). This is in complete agreement with the experimental indications (Webb and Knowles, 1975) that, in addition to a carboxylate base that abstracts the substrate proton, there is an electrophilic component in the isomerase-catalyzed reaction that polarizes the carbonyl group in ES and results in a more facile enolization step.

In Table I we have collected the values of the efficiency function for the various states of the system. For triosephosphate isomerase all three types of change contribute to the improvement of catalytic efficiency, but the greatest improvement results from the relatively unsophisticated method of uniform binding.

Before leaving the question of the efficiency of triosephosphate isomerase, we can ask how the efficiency function for this enzyme varies with the substrate concentration a°; this is shown in Figure 10. On the left of this diagram the efficiency function is constant. This is because the state of lowest free energy is, for small values of a°, always the state e + A. On the right of the diagram, where a° is larger, an intermediate (e.g., e·A in Figure 3) is of lower free energy than e + A, and the enzyme becomes less efficient, since it is tied up as the relatively stable e·A complex. The ambient substrate concentration for the isomerase is indicated by the arrow in Figure 10 and is on the edge of the efficiency plateau. This again shows how finely tuned the free-energy profile of triosephosphate isomerase is to the substrate concentration present in the cytoplasm.

General Discussion

Constraints on Enzyme Efficiency. It was earlier pointed out that individual enzymes may operate in vivo under different constraints. For some purposes, it may be appropriate for the enzyme to work at constant flux over a range of ambient substrate concentrations, while for other systems it will be advantageous for the enzyme to maintain the concentration of the substrate pool relatively constant by varying the flux. It appears likely that enzymes that operate inside the cell mostly belong in the second class, while extracellular systems may fall into the first. Thus the enzymes outside the cell (such as the depolymerases and many hydrolases, e.g., the pancreatic en-

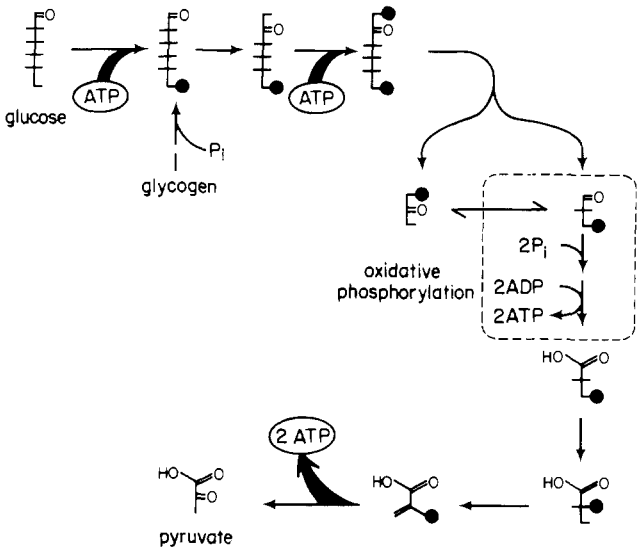

FIGURE 11: The use of phosphoryl groups as "handles" for the binding of glycolytic metabolites. Solid circles represent phosphoryl groups.

zymes in mammals or the extracellular phosphatases of bacteria) may face dramatic changes in substrate levels, and yet be required to maintain a steady flow of feedstock material for adsorption and use by the cell. This constraint is easily met, since a low K_m value will ensure that the extracellular system operates mostly under saturation conditions and very simply maintains a constant flux of products. Intracellular enzymes face a different problem, however, since (at least for materials freely diffusing in the cytoplasm that are not subjected to compartmentalization) sudden or large changes in the concentration of intermediary metabolites are likely to be very undesirable. For instance, even if substantial changes in the levels of such materials as citrate or ATP could be tolerated by all the enzymes involved in their production and utilization (and this is unlikely), there would be consequential effects on the cation levels (e.g., that of Mg^{2+}) which would surely make metabolic control difficult if not impossible. It seems, indeed, that the concentration levels of intracellular substrate pools remain pretty constant, and that metabolic control is exercised by allowing wide variations in the flux of material through the various pathways. The viability of the cell in a changing environment is maintained by ensuring that the control of the flux through the metabolic pathways is sensitive enough to be effected by relatively small changes in metabolite levels (see e.g., Newsholme and Start, 1972).[5] For nonregulatory enzymes that maintain their substrates at or near to equilibrium, therefore, we expect that the ambient substrate levels will be below saturation and the enzyme-catalyzed reactions will be close to second order. This is certainly true for the nonregulatory enzymes in the glycolytic pathway, which is probably the most fully studied sequence in intermediary metabolism (Hess et al., 1969; Hess, 1973).

A second type of alternative for an enzyme in a metabolic pathway is whether it operates under reversible or irreversible conditions. As mentioned above, nonregulatory enzymes whose function is purely catalytic maintain their substrates near to equilibrium; i.e., they operate under reversible conditions. Only those catalysts that work under irreversible conditions are in a position to affect (and therefore maybe control) the flux of material through the pathway. While no enzyme that maintains substrate equilibrium can be a control enzyme, it is not axiomatic that all those enzymes which operate under irreversible conditions are under metabolic control. In a highly developed and evolutionarily perfected pathway, it will be true that only the enzymes at control points will be those working irreversibly, but since the extent of evolutionary development is not known a priori, an efficiency function must be derived without prejudice to the reversibility of the enzyme under scrutiny.

The efficiency function as derived (eq 11) and the dependence of the function y on the four variables of enzyme concentration, reaction velocity, and the concentrations of substrate and product (eq 8) provide a rational basis for quantitatively assessing the efficiency of an enzyme as a catalyst. Once all the rate constants and the ambient substrate concentration for the reaction are known, then y, y_{min} (and therefore E_f) may be calculated. Obviously, any system that falls short of perfection ($E_f < 1$) must either be susceptible of further evolutionary refinement, or have sacrificed simple catalytic efficiency to the possibly higher good of control. Some ways in which this may occur have been elegantly discussed by Newsholme and Start (1972). A system for which E_f is close to unity, however, must have reached the end of its evolutionary development. The efficiency can only be raised further if the enzyme (and one or more of its neighbors in the metabolic sequence) becomes organized in space, by the formation of a multienzyme aggregate. In this case, the diffusional limitation is removed, and y_{min} as defined by eq 10 becomes irrelevant. Evidently this solution is one that nature sometimes prefers, and the existence of multienzyme complexes is well known (Ginsburg and Stadtman, 1970), but the flexibility provided by the multiple branching and interconnections of metabolic pathways, where a given metabolite may be shared amongst several pathways, apparently leaves the majority of intracellular enzymes as independently diffusing catalysts in the cytoplasm.[6]

Improvements in Enzyme Efficiency. Earlier in this paper, we postulated three types of change in the free-energy profile that the evolutionary development of an enzyme could effect. In order of increasing subtlety, these were called: uniform binding, differential binding, and catalysis of elementary steps. *Changes in uniform binding affect all the internal states (intermediates and transition states) equally* and, in addition to the rationale provided earlier, we suggest that such changes are made especially easy if all the substrate species have a "handle" that facilitates changes in uniform binding. It is tempting to suggest that, in the glycolytic pathway, the substrate phosphoryl groups provide such a handle. As shown in Figure 11 in the first committed step of glycolysis, glucose 6-phosphate is produced either via the phosphorolysis of gly-

[5] Thus the concentrations of such glycolytic intermediates as dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, 3-phosphoglycerate, and phosphoenolpyruvate change by only 2-6-fold when electrical tetanization increases the flux through the pathway by more than 1000-fold (Hohorst et al., 1962). Similar results are evident from studies on insect flight muscle (Sacktor and Wormser-Shavit, 1966).

[6] There have been various reports of the loose association of glycolytic enzymes with each other and with subcellular particulate elements such as F-actin or the inner surface of the erythrocyte membrane (for references, see Foemmel et al., 1975; Clarke and Masters, 1975; Tillman et al. 1975). But by no means all of the glycolytic enzymes are so bound, and a mixture of purified glycolytic enzymes is "almost identical" with intact muscle tissue in respect of glycolytic metabolism (Scopes, 1974). The very recent suggestion of Mowbray and Moses (1976), that for an extract from ruptured *E. coli* spheroplasts there may be some compartmentation of glycolytic intermediates, raises a number of interesting questions which will be considered elsewhere.

cogen or by phosphorylation of glucose. This phosphoryl group serves no chemical function and is retained during the conversion of glucose 6-phosphate to fructose 6-phosphate. Now, immediately *before* the aldol cleavage of the hexose to two trioses, a further ATP is consumed to yield fructose 1,6-bisphosphate. This phosphorylation means that each of the trioses produced by the aldolase cleavage is phosphorylated. The next reaction is that catalyzed by glyceraldehyde-3-phosphate dehydrogenase and is the only step of the glycolytic pathway in which oxidative phosphorylation occurs, the acyl phosphate product then being used to synthesize ATP. The subsequent conversions of the pathway then occur, and only in the *last* step (that catalyzed by pyruvate kinase) are the phosphoryl group "handles" removed (regenerating ATP, which may be considered as the quid pro quo for the two ATP's used in putting the "handles" on higher up the pathway). This view may be dangerously teleological, but it does attractively accommodate the otherwise puzzling sequence of phosphorylation and dephosphorylation in glycolysis.[7] As has been discussed with reference to triosephosphate isomerase, there is an immediate and substantial catalytic advantage in optimizing uniform binding, and one may speculate that this advantage caused the early selection of metabolic intermediates with handles appropriate for the facile adjustment of uniform binding. [An alternative view is that the existence of phosphoryl groups provides the possibility of larger free energies of *intrinsic* substrate binding than could be easily realized in their absence, thereby allowing the utilization of this free energy for catalytic purposes. This view, and mechanisms by which this utilization could occur, have been discussed by Jencks (1975).]

Changes in the internal thermodynamics must be more discriminating, and mutations that result in enzyme structures capable of such detailed recognition would presumably be more rare. As has been shown, the important changes in internal states require the catalyst to differentiate between two intermediates that are connected by a single elementary step. In the case of triosephosphate isomerase, for example, this requires that the enzyme differentially binds (say) dihydroxyacetone phosphate and the enediol derived from it, even though these two species differ only slightly in geometry and in the position of one or two protons. While there is no doubt that such discriminatory recognition is perfectly feasible for a biological system (the phenomenon of antibody specificity amply testifies to the capabilities of proteins to make nice distinctions), it is also likely that such discrimination is more difficult to achieve than uniform binding. The mechanisms for discrimination are limited: hydrophobic interactions are less probable unless the van der Waals constraints of the active site are very finely tuned, electrostatic interactions will also be rather nonspecific unless the two intermediates in question differ markedly in charge distribution, and hydrogen bonds (the specificity of which may derive more from length than from angle, as discussed by Fersht, 1974) are perhaps the most likely interactions to achieve the necessary changes in internal thermodynamics.

Finally, if uniform binding has been optimized, and if the internal thermodynamics of the connected kinetically significant intermediates has been equalized, then *the enzyme may only improve further by reducing the activation free energy of the kinetically significant elementary step.* This requires the development of the catalytic apparatus of the enzyme either to create localized centers of high reactivity and to utilize multifunctional catalysis, and/or to stabilize the transition state for the kinetically significant step. The consequences of uniform binding and changes in the internal thermodynamics make distinctions between the destabilization of enzyme–substrate ground states (Vallee and Williams, 1968) and the stabilization of the highest transition state (Wolfenden, 1972; Lienhard, 1973) unnecessary since, whatever catalytic devices operate to reduce the free energy of activation of the elementary step, the uniform binding criterion ensures that the levels of the important internal states are optimized with respect to the external states.

Evolution to Perfection. For triosephosphate isomerase, we have shown that the catalyzed reaction has a value of E_f near to unity; that is, this enzyme is as efficient as it can be for a system free in solution. Qualitatively, it is evident that, since all the intermediates are of higher free energy than that of the state for [enzyme + predominant substrate], and since the highest free energy barrier is that representing the binding of the less stable substrate to the enzyme, there is no catalytic advantage to be gained by further adjustment of the free-energy levels of the intermediates, or of those transition states that do not relate to diffusion processes. Is this enzyme a rare system, or have many enzymes reached catalytic perfection? Not surprisingly, the answer seems to be that many enzymes have reached the end of their catalytic development. Two arguments support this view. First, values of k_{cat}/K_m for a number of enzyme-substrate systems are in the region of 10^8 to 10^9 M^{-1} s^{-1}. Since k_{cat}/K_m is the second-order rate constant for what is effectively the bimolecular reaction between enzyme and substrate, and numerical values of 10^8 to 10^9 M^{-1} s^{-1} are those expected for the diffusion limit, then *as long as* the *in vivo* substrate concentration is somewhat below K_m (i.e., that the constraints on the system are that it should work below saturation: see above), the enzyme is under diffusion control. The covalency changes that constitute "catalysis" by such enzymes must have been accelerated to the point where they have little or no effect upon the flux. Secondly, the steady-state rates of catalysis by a number of enzymes are known to be limited by the rate of loss of product from the enzyme. This has been shown for some dehydrogenases (see, e.g., Orsi and Cleland, 1972; Heck et al., 1968) and a number of other systems (Cleland, 1975). It is not uncommon, therefore, that substrate binding steps (i.e., on-off steps) are rate limiting, and this requires that the actual catalytic steps for these enzymes are relatively fast. It is worth stressing here that, if a reaction is diffusion controlled in one direction, then as long as the ambient substrate concentration is below K_m, the rate of the reverse reaction is limited by the separation of product from the enzyme, and this situation *must* correspond to the maximum achievable rate of reaction.

One final point should be made about the free-energy profile of the triosephosphate isomerase reaction. The free-energy levels of the transition states for the catalytic steps are only *just* lower than the unerodable barrier that represents the loss of the less stable product from the enzyme. Further, the probable free-energy levels for the reaction intermediates are only just higher than that representing the state [enzyme + predominant substrate] (see Figure 8). These features may well be inevitable consequences of the evolution of catalytic function. Consider a catalyzed reaction for which one free-energy barrier is much higher than all the others. Assuming a selective pressure exists to improve the flux between substrate and product, then mutational events that result in the lowering of this rate-limiting transition state will be selected for. Every time that the barrier

[7] Indeed, Davis (1958) pointed out a number of years ago that *all* "the known low molecular weight and water-soluble biosynthetic intermediates possess groups that are essentially completely ionized at neutral pH".

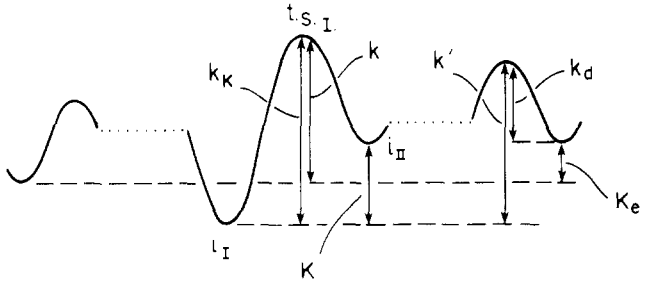

FIGURE 12: Illustration of the effect of differential binding of connected intermediates (i_I and i_{II}).

is lowered by 1.4 kcal/mol, the enzyme becomes ten times more effective. At the point where the barrier has been lowered to the level of some *other* transition state in the multistep reaction, then the most that can be gained in rate terms by lowering the energies of either of the two transition states is twofold. We may expect, then, to find that reaction intermediates in refined systems will partition rather evenly between substrates and products. Further, as soon as the erodable transition states lie just below the unalterable diffusion barrier (transition state 4 in Figure 8), and the intermediates have been just destabilized enough, no further selective pressure to improve the catalytic efficiency will be exerted. In terms of catalysis, the enzyme will have reached the end of its evolutionary development.

Acknowledgments

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Appendix

The form of the free-energy relationship will be:

$$\ln k_K = \ln k_{K=1} + \beta \ln K \qquad \text{(i)}$$

From the free-energy differences shown in Figure 12 and their associated rate constants, we may also write:

$$\ln k_K = \ln k - \ln K_e - \ln k_d + \ln k' \qquad \text{(ii)}$$

On the basis that the uniform binding condition has been met, then from eq 12 (and cf. Figure 6), we have the further relation:

$$\frac{1}{k} = \frac{1}{k'}(1 + K) \qquad \text{(iii)}$$

Eliminating k' and k_K from eq i, ii, and iii gives:

$$2 \ln k - \ln K_e - \ln k_d - \ln k_{K=1} = \beta \ln K - \ln (1 + K) \qquad \text{(iv)}$$

In this equation, K_e, k_d, and $k_{K=1}$ are constants, and we are interested in how k varies with changes in K. Differentiation of eq iv shows that k will be a maximum (and hence also the efficiency function will be a maximum), when

$$K = \frac{\beta}{1 - \beta} \qquad \text{(v)}$$

Typical values of β for an elementary step lie between 0.3 and 0.7, which give values of K between 0.4 and 2.5. These values of K are close enough to unity for the transition state to be essentially symmetrical. Hence to optimize the efficiency for a single step, $K \rightarrow 1$, $\beta \rightarrow 0.5$ and the free energies of i_I and i_{II} will become equal. Equation iv then becomes

$$2 \ln k = \ln k_{K=1} + \ln K_e + \ln k_d - \ln 2 \qquad \text{(vi)}$$

The qualitative reason for this is that efficiency is improved by lowering i_{II} (and with it, t.s.$_I$). The uniform binding criterion then raises the three states, i_I–t.s.$_I$–i_{II}, up, thereby reducing the free energy associated with k' (Figure 12). These changes can continue until i_{II} becomes level with i_I.

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