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Perfection in Enzyme Catalysis: The Energetics of Triosephosphate Isomerase

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During the last 10^9 years, the structures of biological macromolecules have been refined and improved so as to optimize the functioning of the organisms that use them. Enzymes are more specific and more effective than the simple molecular units of which they are made, and compared to simple catalysts (e.g., acetic acid or imidazole), the free-energy barriers for an enzyme-catalyzed transformation are much lower, allowing the organism to synthesize less catalyst to produce a given flux of substrate.

Can we say how far this process has gone? For some enzymes, there is going to be a conflict between changes that maximize catalytic effectiveness and changes that optimize control, and for these enzymes we may expect that some catalytic power has been sacrificed to the higher good of metabolic control.[1] On the other hand, very many enzyme systems appear not to be involved in metabolic control (at least not at the level of governing the flux of substrate through a metabolic pathway), and for these we can ask to what extent the selective pressures of evolution have produced the perfect catalyst.

In this Account, we shall suggest one measure of perfection and further show how a combination of mechanistic approaches using isotope methods allows the essentially complete description of the energetics of the reaction catalyzed by triosephosphate isomerase from muscle. This enzyme appears to have arrived at the end of its evolutionary development as a catalyst.

In muscle, adenosine triphosphate (ATP) is produced (at least partially) by the anaerobic conversion of muscle glycogen to lactate. Glycogen is converted in a number of steps to fructose 1,6-bisphosphate, and these conversions consume 1 mol of ATP per mol of glucosyl unit (Figure 1). Fructose 1,6-bisphosphate is then cleaved by aldolase into two three-carbon units, dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP), and only GAP is further catabolized to lactate. Each mole of GAP that is converted to lactate yields 2 mol of ATP.

From Figure 1 it is clear that, without triosephosphate isomerase (TIM), which allows the utilization of DHAP, muscle glycolysis would produce only one ATP for every glucose unit. Yet with the isomerase, all six carbon atoms of fructose 1,6-bisphosphate can be utilized, so that the net yield of ATP is then 3 mol per mol of glucose unit.

Since in the absence of TIM only one ATP would be produced, it is reasonable to suppose that the evolutionary pressure for an efficient isomerase would have been intense, since for "flight or fight" there is an instant requirement for muscle ATP. We expect, therefore, that TIM may be a good candidate for a very efficient enzyme, both in the rabbit and in the fox that pursues it.

The Energetics of the Isomerase-Catalyzed Reaction

We shall now describe a number of experiments on which the derivation of the free-energy profile for the triosephosphate isomerase reaction is based. Only experiments that are easily discussed nonalgebraically will be considered, in order to provide a qualitative view of the way in which the different types of isotope experiment provide different kinds of kinetic information.

TIM is a dimeric protein[2] of subunit molecular weight 26 500[3] and requires no cofactors or metal ions for

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(1) E. A. Newsholme and C. Start, *Handbook of Physiol., Sect. 7: Endocrinol.*, 1, Chapter 23 (1972).

(2) There is no evidence that the subunits of TIM behave cooperatively, or that TIM has any control function: the control points in glycolysis appear to be at phosphorylase, phosphofructokinase, and pyruvate kinase.

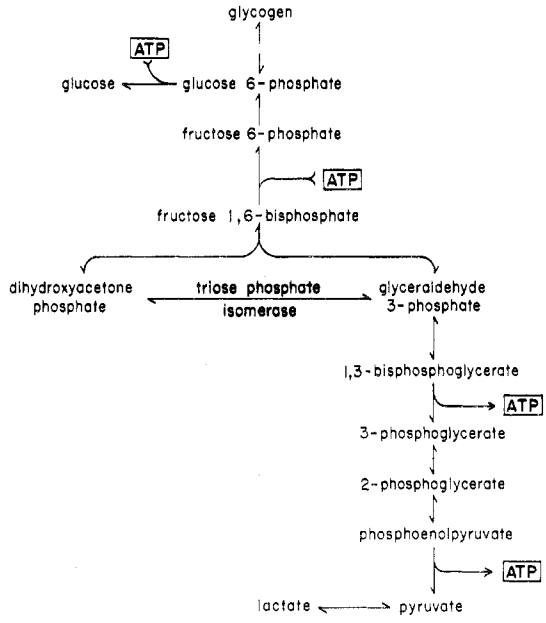

Figure 1. Muscle glycolysis.

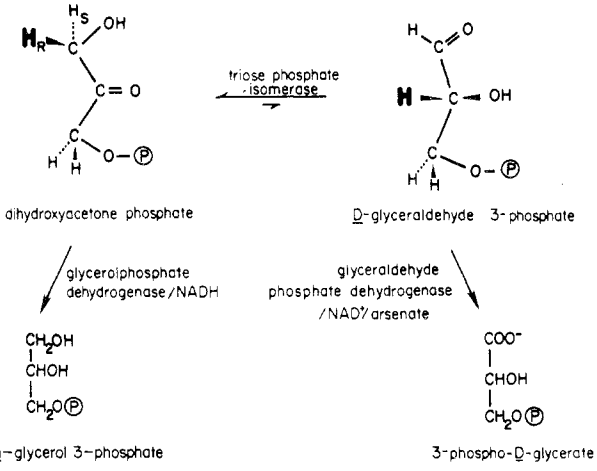

Figure 2. The reaction catalyzed by triose phosphate isomerase. The hydrogens labilized in the catalyzed reaction are shown in heavy type.

maximal activity. The catalyzed reaction is chemically simple (Figure 2), and the early studies of Rose and his collaborators[4] elegantly showed that it has a number of important advantages for mechanistic study. The overall equilibrium constant[5] is near enough to 1 to allow the catalyzed reaction to be studied in either direction. As will be seen, this possibility provides new information since effective rate- and product-determining steps are different for the forward and reverse

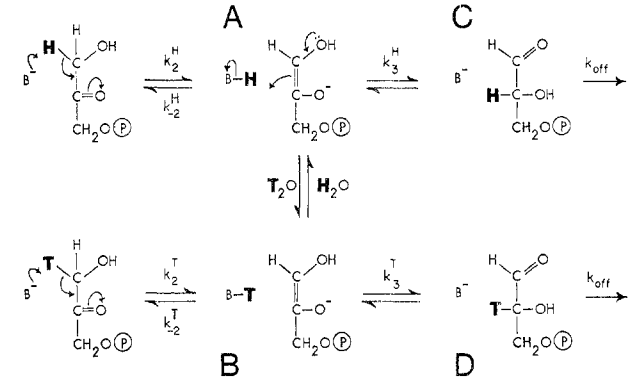

Figure 3. Mechanistic pathway for the triosephosphate isomerase catalyzed reaction. All species are enzyme bound.

reactions. Nature has provided the appropriate dehydrogenases for running the reaction in either direction under "irreversible conditions", i.e., where the free product is removed as fast as it is formed by isomerase (Figure 2). The isomerase-catalyzed reaction is fast, the k_{cat} values being near 500 s^{-1} (Figure 2, left to right) and 5000 s^{-1} (Figure 2, right to left).[3]

For structural work too, the system has attractive features. Since the reaction involves a single substrate and a single product, the addition of either substrate to enzyme will result (in an enzyme crystal, for example) in the formation of the lowest energy enzyme–triose phosphate complex.[6] The possibility is therefore open for the study both of native enzyme and of a *productive* complex between enzyme and natural substrate, which is something that has eluded crystallographers until very recently. This possibility is being exploited by Phillips and his group, who are solving the crystal structures of enzyme and of enzyme–substrate complex independently,[7] and also augurs well for spectroscopic studies on the enzyme–substrate interaction.

Transfer. In the isomerase-catalyzed reaction, the hydrogen on C-2 of D-GAP and the *pro-R* hydrogen on C-1 of DHAP are labilized. If specifically labeled [1-(*R*)-^{3}H]DHAP is isomerized by TIM in the presence of glyceraldehyde-phosphate dehydrogenase, NAD^+, and arsenate (i.e., under irreversible conditions; Figure 2), the resulting 3-phosphoglycerate (and therefore the immediate product, GAP) has 3 to 6% of the specific radioactivity of the starting DHAP.[8] There is thus a little transfer of tritium from C-1 of DHAP to C-2 of GAP, the hydrogen of the new carbon–hydrogen bond being very largely derived from the solvent. Since neither substrate nor product exchanges tritium with the solvent (at any significant rate) in the absence of enzyme, there must be an intermediate in the enzyme-catalyzed reaction that *can* exchange, rapidly. Accordingly, Rose[4] proposed an attractive mechanism involving proton transfers and an enzyme-bound enediol intermediate in rapid equilibrium with the solvent (Figure 3). The almost complete loss of tritium from

(3) S. J. Putman, A. F. W. Coulson, I. R. T. Farley, B. Riddleston, and J. R. Knowles, *Biochem. J.*, **129**, 301 (1972); D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, C. I. Pogson, and I. A. Wilson, *Nature (London)*, **255**, 609 (1975).

(4) S. V. Rieder and I. A. Rose, *J. Biol. Chem.*, **234**, 1007 (1959); I. A. Rose, *Brookhaven Symp. Biol.*, **15**, 293 (1962).

(5) K_{eq} = total[DHAP]/total[GAP] = 22. However, only 4% of GAP in aqueous solution is the unhydrated aldehyde substrate, and 59% of DHAP is the unhydrated ketone substrate. This makes K_{eq} for the *unhydrated* species about 340. As long as the hydrates do not inhibit the catalyzed reaction (and there is NMR evidence that they bind poorly if at all), then the K_m values for the substrates can simply be adjusted by the appropriate ratio [see D. R. Trentham, C. H. McMurray, and C. I. Pogson, *Biochem. J.*, **114**, 19 (1969); S. J. Reynolds, D. W. Yates, and C. I. Pogson, *ibid.*, **122**, 285 (1971); G. R. Gray and R. Barker, *Biochemistry*, **9**, 2454 (1970)].

(6) Or, conceivably, an enzyme–intermediate complex, if this were the most stable.

(7) D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, and C. I. Pogson, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 151 (1971); D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, C. I. Pogson, and I. A. Wilson, *Nature (London)*, **255**, 609 (1975).

(8) J. M. Herlihy, S. G. Maister, W. J. Albery, and J. R. Knowles, *Biochemistry*, **15**, 5601 (1976). The observed extent of tritium transfer depends upon the extent of the reaction.

Type of Experiment	Direction of Experiment	Steps Involved 1	2	3	4	Note
Isotopic Discrimination in product	→				⌒	a
	←		⌒			b
Exchange versus Conversion	→		⌒		⌒	c
	←		⌒		⌒	d

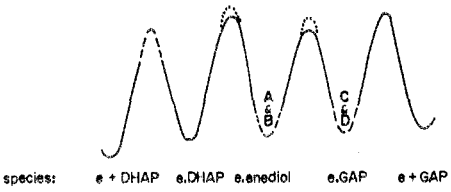

Figure 4. Schematic representation of information forthcoming from experiments in tritiated water. The dotted lines in the profile represent the effect of tritium rather than hydrogen transfer, and are shown as transition-state differences rather than ground-state differences simply for clarity. (a) The $k_3^H >> k_3^T$ inequality is irrelevant, since C ⇌ A ⇌ B ⇌ D (see Figure 3), and the loss of GAP from e·GAP is relatively slow. (b) The $k_{-2}^H >> k_{-2}^T$ inequality *is* seen, since the loss of DHAP from e·DHAP is relatively fast. (c) At the start of the reaction, the relevant barriers are as shown, since "exchange" is measured by the appearance of *tritium* in the starting DHAP, and "conversion" is d[GAP]/d*t*, which is determined by the loss of GAP from e·GAP. For the situation later in the reaction, see the text. (d) Here, "exchange" is measured by the appearance of tritium in starting GAP which is determined by the loss of GAP from e·GAP, and "conversion" is d[DHAP]/d*t*, which is determined by the flux of material over the lower barrier of step 2.

the 1*R* position of DHAP indicates that A and B in Figure 3 are essentially at equilibrium.

Discrimination. When the radiolabel is in the solvent and not in the substrate DHAP, it is found as expected that the product GAP is radioactively labeled on C-1, as it must be since A and B are essentially at equilibrium. More importantly, however, the specific activity of the product is close to that of the solvent, a small (1.3-fold) discrimination against tritium being observed.[9] That is, the enediol shows very little discrimination between ^1H and ^3H when it picks up hydrogen at C-2. Since k_3^H must be larger than k_3^T (Figure 3) by 6–20-fold (which is the expected range of primary tritium kinetic isotope effects), the rate-determining transition state must come *after* this proton-transfer step, and the $k_3^H >> k_3^T$ inequality is made irrelevant by the fact that A, B, C, and D (Figure 3) are all essentially at equilibrium. The most logical slow step is then that involving the loss of GAP from the enzyme–GAP complex (k_{off}).

At this point, we can take advantage of the possibility of looking at the reverse reaction. With unlabeled glyceraldehyde 3-phosphate as substrate, under irreversible conditions in tritiated water, the specific activity of the product α-glycerophosphate (which is the same as that of the immediate product, DHAP: Figure 2) is only about 11% that of the solvent in which the reaction is run.[10] This 9-fold discrimination against tritium when GAP is the substrate agrees with the expected inequality of the constants $k_{-2}^H >> k_{-2}^T$ (Figure 3), which *does* now result in a low specific radioactivity of the product. The rate of loss of DHAP

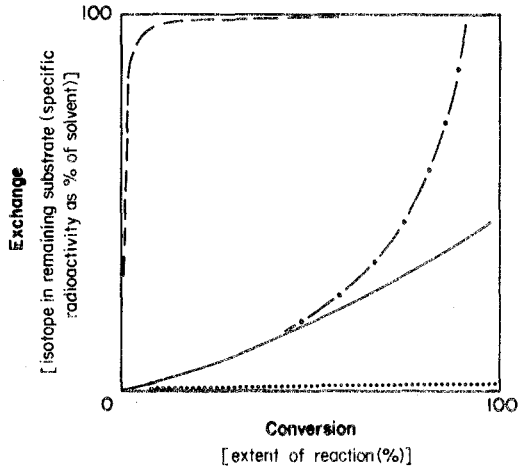

Figure 5. Exchange vs. conversion plots for the reaction catalyzed by triosephosphate isomerase. (– – –) Expected line for rapid preequilibrium between substrate and exchanging intermediate; (· · ·) expected line for very rapid conversion of exchanging intermediate to product; (—) experimental line where glyceraldehyde phosphate is the substrate (Figures 2 and 3, right to left); (– · – · –) experimental line where dihydroxyacetone phosphate is the substrate (Figures 2 and 3, left to right).

from the enzyme–DHAP complex must therefore be faster than the product-forming proton-transfer steps k_{-2}^H and k_{-2}^T. [In other words, when DHAP is the substrate and the reaction is run from left to right, the product-forming step (step 3 in Figure 4) is not the same as the rate-limiting step (step 4 in Figure 4). Yet when GAP is the substrate and the reaction goes from right to left, the product-forming step (step 2 in Figure 4) is the *same* as the rate-limiting step.]

Simply on the basis of isotope transfer and isotope discrimination experiments, therefore, we obtain a qualitative overall picture for the relative heights of the free energy barriers of the TIM-catalyzed reaction (see Figure 4; barrier 1 must be lower than barrier 2, and barrier 3 must be lower than barriers 2 and 4). We have here, however, a situation that can be further exploited. Neither substrate exchanges isotope with the solvent, but an enzyme-bound intermediate does. What we can do, in effect, is to feed isotope from the solvent into the middle of the reaction pathway (at the stage of the rapidly exchanging intermediate A ⇌ B) and follow the isotope to see how this enzyme–enediol intermediate partitions between starting material and product. This is a classical type of physical-organic experiment, notably used by Bender in his demonstration of the tetrahedral intermediate in ester hydrolysis in $H_2{}^{18}O$.[11] The application of this principle to the TIM reaction is described below.

Exchange vs. Conversion. In exchange–conversion experiments, the reaction is run under irreversible conditions[12] with initially unlabeled substrates in tritiated water and quenched at different extents of conversion of substrate to product. The remaining substrate and the product are separated and their specific radioactivities determined. The specific radioactivity of remaining GAP (when the reaction is run in the direction GAP → DHAP ⟹ glycerophosphate,

(9) S. G. Maister, C. P. Pett, W. J. Albery, and J. R. Knowles, *Biochemistry*, **15**, 5607 (1976).
(10) S. J. Fletcher, J. M. Herlihy, W. J. Albery, and J. R. Knowles, *Biochemistry*, **15**, 5612 (1976).
(11) M. L. Bender, *J. Am. Chem. Soc.*, **73**, 1626 (1951).
(12) It is crucial that this be checked: any equilibration of substrate by the target enzyme (because of the inadequacy of the coupling enzyme) would of course vitiate the results.

Figure 2) is found to increase with the extent of the reaction as shown in Figure 5 (full line). One can see from the initial gradient of the line that the enediol intermediate (A ⇌ B in Figure 3) undergoes "conversion" to dihydroxyacetone phosphate about three times faster than it suffers "exchange" (picking up tritium to return to [^{3}H]glyceraldehyde phosphate). Fortunately the partitioning of the intermediate is not very much in favor of starting material (in which case we should have seen the dashed line in Figure 5), nor very much in favor of product (in which case we should have seen little or no radioactivity appearing in remaining starting material: the dotted line in Figure 5), but is balanced finely enough between the two for the partition ratio to be determined quantitatively.[10]

The difference in barrier heights that is being measured here is shown in Figure 4d. Now, if the reaction is run in the left-to-right direction with initially unlabeled dihydroxyacetone phosphate as substrate in tritiated water, the exchange–conversion graph takes on a more interesting form (see Figure 5, dot–dash line).[9] First, it appears that early in the reaction the enediol intermediate is converted to glyceraldehyde phosphate about three times faster than it suffers exchange (picking up tritium to return to [^{3}H]dihydroxyacetone phosphate). The difference in barrier height being measured is shown in Figure 4c. Indeed, the fact that the partitioning of the enediol in experiment Figure 4c is 1:3 and that in Figure 4d is 3:1 means that the difference between the rates of step 2 for ^{1}H and ^{3}H is: $3 \times 3 = 9$-fold. This agrees nicely with the same difference (9-fold) determined from the discrimination experiment (see Figure 4b). That is (even though the arguments are here rather oversimplified for the sake of a useful physical picture), the two exchange-conversion experiments combine to agree with one of the isotopic discrimination experiments.

As the reaction of dihydroxyacetone phosphate proceeds, what remains becomes more and more heavily labeled, and the specific activity rises rapidly toward the end of the reaction. This is readily explicable by reference to Figure 4. Labeled DHAP is formed by exchange back over step 2, and the remaining substrate becomes a mixture of protonated and tritiated DHAP. Now, the unlabeled DHAP will be consumed faster than the labeled because of the isotope effect inherent in the hydrogen abstraction step 2 ($k_2^H >> k_2^T$, Figure 3). That is, the tritiated DHAP formed by exchange lasts longer than the original unlabeled substrate. This leads to the curve in Figure 5, the exact form of which is determined by the partitioning ratio of the intermediate and the size of the kinetic isotope effect for reaction (from left to right) in step 2.

Deuterium Kinetic Isotope Effects. Experiments with tritium are normally competitive: labeled molecules compete with unlabeled ones and thus allow the investigation of product-forming steps which may not be rate limiting in the overall reaction. In contrast, the use of deuterium-labeled substrates provides information about the kinetic importance of protonation and deprotonation steps in the overall process, thereby both complementing studies with trace amounts of tritium and providing a link between the details of individual steps from the tritium work and the steady-state kinetic parameters for the reaction of unlabeled material.

In the present case, the use of specifically deuterium labeled substrates provides unusually helpful information. Consider the reaction of D-[2-^{2}H]glyceraldehyde 3-phosphate (Figure 4, right to left). Substrate binds to the enzyme (step 4) and the 2-^{2}H atom is removed in step 3 to give the enediol intermediate. But we know that bound enediol and bound GAP are in equilibrium with each other and with the solvent (A ⇌ B ⇌ C ⇌ D), so the deuterium will be rapidly washed out into the solvent (the system becoming unlabeled) before the rate-limiting collapse of the enediol in step 2 by *hydrogen* (not deuterium) transfer. The deuterated substrate thus loses all its label before the rate-limiting conversion to product, and we expect to see no isotope effect in either k_{cat} or K_m. None is observed,[13] and this is probably the clearest confirmation of the qualitative correctness of Figure 4.

When [1(*R*)-^{2}H]dihydroxyacetone phosphate is the substrate (Figure 4, left to right), binding to the enzyme is followed by a relatively slow abstraction of the 1(*R*) deuterium by the enzyme giving the bound enediol. Essentially all the ^{2}H is lost at this stage by equilibration with solvent ^1H_2O, and this is the irreversible step for the reaction of the ^{2}H compound. The observed rate of reaction is measured by the rate of GAP formation, which is about one in four of the molecules arriving in the enediol–GAP "pool".

Since the isotopic content of this pool depends only on the solvent, the partitioning of the molecules *out* of the pool will be the same, whichever isotope they started with in the 1(*R*) position. The relative rate of reaction of labeled and unlabeled DHAP molecules is thus governed only by the flux of molecules *into* the pool (over step 2). This leads to the unusual state of affairs that, in observing the steady-state rate difference between labeled and unlabeled DHAP, one seems to measure the full primary kinetic isotope effect of a step that is not rate determining for the overall reaction! Experimentally, a kinetic isotope effect with [1(*R*)-^{2}H]DHAP is seen, with $k_{cat}^H/k_{cat}^D = 2.9$.[13,14]

Treatment of Results. The above discussion has focused upon those aspects of the isotope experiments that are most amenable to nonalgebraic description in order to show some of the ways in which the energetics of the reaction can be defined. In all, four kinds of experimental situation have been studied: (1) Specifically tritiated substrate in ^1H_2O, the specific radioactivity of product (transfer) and of remaining substrate (enrichment or depletion) being measured as a function of the extent of reaction; (2) unlabeled substrate in tritiated water, the specific radioactivity of remaining substrate (exchange) and of product (discrimination) being measured as a function of the extent of reaction; (3) measurement of the initial steady-state rates for specifically deuterated substrates in ^1H_2O; and (4) measurements of the steady-state rates for unlabeled substrates in ^1H_2O. No assumptions are made about

(13) P. F. Leadlay, W. J. Albery, and J. R. Knowles, *Biochemistry*, **15**, 5617 (1976).

(14) The apparent discrepancy between the deuterium and the tritium isotope effects for step 2 (the values of 2.9 and 9 do not obey the Swain relationship: C. G. Swain, E. C. Strivers, J. F. Reuwer, and L. J. Schaad, *J. Am. Chem. Soc.*, **80**, 5885 (1958)) arises because there is incomplete exchange of isotope with the solvent at the enediol stage. This means that 2.9 is slightly lower than the actual deuterium isotope effect for step 2, and 9 is slightly larger than the product tritium discrimination would be if the enediol had equilibrated *completely* with solvent. For a more complete discussion, see ref 10, 13, and 15.

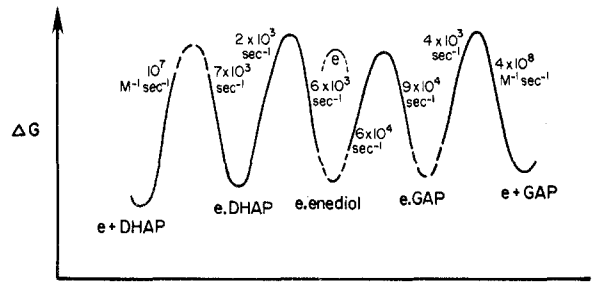

Figure 6. Free-energy profile for the triosephosphate isomerase catalyzed reaction. The dashed parts of the profile are less precisely established, and the rate constants for the elementary steps involving these states are lower limits. However, these states are kinetically insignificant, and their uncertainty does not affect the conclusions presented here. The barrier marked e is that for the exchange of protons between the enzyme–enediol and the solvent.

which steps are fast (not even that the exchange reaction A ⇌ B is fast), yet the results from the above experiments can be analyzed to obtain the rate constants for all the kinetically significant steps in the catalyzed reaction.[15] This general analysis is applicable to other analogous enzyme-catalyzed reactions.

The result of the analysis[15] for the TIM-catalyzed reaction is shown in Figure 6.[16] In drawing Figure 6 we have to set a standard state in order that bimolecular steps and unimolecular steps can be represented in the same diagram. Since we are interested in the operation of TIM in vivo, the proper thing to do is to take the *actual* in vivo level of substrate as the standard state. The concentration of triose phosphates in muscle is around 40 μM,[17] and this is the standard state for Figure 6.

The free-energy profile of Figure 6 provides the answers to a number of questions and poses some new ones. First, we can see that the crystal structure determination being carried out on the TIM–substrate complex[6] will presumably be largely that of the enzyme–DHAP complex, since this is thermodynamically the more stable of the two enzyme–substrate complexes.[18] Secondly, the problem of enzyme catalysis can now be posed at the level of the elementary step. In the present case, it is known that the rate of enolization of DHAP in aqueous buffer (pH 7.0, 30 °C) is 6×10^{-7} s^{-1} (ref 19), and the same process at the active site of TIM (pH 7.5, 30 °C) is more than 10^9 times faster, at 2×10^3 s^{-1}. Thirdly, the activation free energy

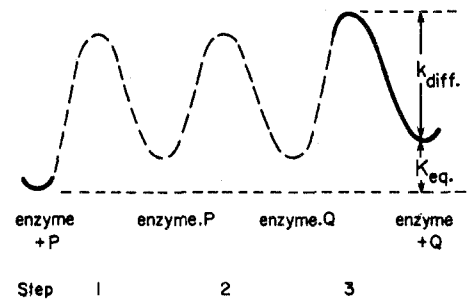

Figure 7. Notional free-energy profile for a "perfect" enzyme that interconverts P and Q.

for the isomerization of GAP catalyzed by the enzyme[20] corresponds to a rate of 4×10^8 M^{-1} s^{-1}, which is that expected for a simple diffusion process.[21] It is this fact, and the observation that the other free-energy "hills" are not much lower than that for the GAP "on–off" step nor are the "valleys" much higher than the free enzyme + free DHAP state, that indicates the TIM has achieved a condition where evolutionary pressure is no longer being exerted upon it.[22]

Perfection

If one set out to design the most effective catalyst for the interconversion of two freely diffusing species, P and Q, at a given concentration, what are the limitations on the P ⇌ Q flux that can be achieved? In principle, there are only two. First, the ΔG_0 for the reaction is immutable, the overall equilibrium constant being determined only by the chemical nature of P and Q. A catalyst (at catalytic concentrations, anyway) cannot affect the position of equilibrium. The second limitation is that the enzyme cannot catalyze the P ⇌ Q interconversion faster than P (or Q) can "find" the enzyme in solution. This just says that there is a diffusion limitation for any bimolecular process in solution. And it is the diffusion limit for the thermodynamically *less* stable substrate that is important, as is seen from Figure 7.

Once the enzyme has (a) lowered the free energies of all transition states below that of step 3 (Figure 7) and (b) raised the free energies of all intermediate states above that for the (enzyme + P) state, then there is nothing more it can do. In evolutionary terms we can see that the transition state for step 3 (Figure 7) cannot be eroded, and that, while the evolutionary pressures of selection will erode the transition states for steps 1 and 2,[23] once these are lower in free energy than that of step 3 there is no rate advantage to be gained by

(15) W. J. Albery and J. R. Knowles, *Biochemistry*, 15, 5588 (1976).
(16) W. J. Albery and J. R. Knowles, *Biochemistry*, 15, 5627 (1976). The use of free-energy profiles begs a number of questions, of course, and is less appropriate for illustrating the energetics of multisubstrate reactions. For an isomerase, however, such a profile can realistically be viewed as a section through the energy surface, and does have illustrative value.
(17) J. R. Williamson, *J. Biol. Chem.*, 240, 2308 (1964), finds triose phosphate levels in heart muscle to be 80 nmol/g (dry weight) which, on the basis that there is 2 mL of cytoplasm/g (dry weight), is ~40 μM. The concentrations of glycolytic metabolites evidently do not change much (i.e., glycolysis is a "constant pool–variable flux" system): levels of the triose phosphates only change by 3–7-fold when electrical tetanization of rat abdominal wall muscle increases the flux through glycolysis by more than 2000-fold (H. J. Hohorst, M. Reim, and H. Bartels, *Biochem. Biophys. Res. Commun.*, 7, 137 (1962)). Analogous results are evident from studies on insect flight muscle (B. Sacktor and E. Wormser-Shavit, *J. Biol. Chem.*, 241, 624 (1966)).
(18) We cannot expect that the crystal-structure determination will be able to distinguish between bound GAP and bound DHAP at 2.5-Å resolution, since there are only slight stereochemical differences, and chemically the only difference is in the position of two protons and two electrons. So it is nice to know what model to build into the map.
(19) A. Hall and J. R. Knowles, *Biochemistry*, 14, 4348 (1975).
(20) Treated as a bimolecular process, i.e., under subsaturation conditions, where k_{cat}/K_m is measured.
(21) G. G. Hammes and P. R. Schimmel, "The Enzymes", Vol. II, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1970, p 67. These authors list enzyme–substrate association rates which lie in the range 10^6–10^8 M^{-1} s^{-1}. Our observed rate is at the upper end of this range of observed values and is close to that expected for such molecules.
(22) It is interesting to note that TIM is likely to have reached its present state at least 400 million years ago, since the coelacanth (which has not changed morphologically in this time) produces TIM of similar specific catalytic activity to that from other species (E. Kolb and J. I. Harris, *Biochem. J.*, 130, 26P (1972)). TIM further shows a very low evolutionary rate of about 2 amino acid substitutions per 100 residues per 100 million years (E. Kolb, J. I. Harris, and J. Bridger, *Biochem. J.*, 137, 185 (1974)).
(23) Step 1 has its unerodable diffusion limit too, of course, but in the example of Figure 7 this may be irrelevant. It is important for the enzyme to receive Q at the diffusion limit, since this is the highest free-energy barrier the system must surmount: P may form the enzyme–P complex less rapidly and still not affect P ⇌ Q flux.

further erosion of steps 1 and 2 and no pressure for selection of a better catalyst.[24] Analogous arguments can be applied to the energies of reaction intermediates: an excessively stable intermediate state will simply tie up valuable enzyme in the energy well.

And so it seems from Figure 6 that triosephosphate isomerase, an enzyme which we have argued will have been under intense pressure during evolutionary time, has actually been refined to a state where it cannot get any better.[25,26]

The Mechanism of the Enzyme. Whatever kineticists or crystallographers may say, a mechanism is more than a bald collection of rate constants and more than an uncompromising structure; it is something of a synthesis of the two, in which chemical significance is given to kinetic events. So far, we have concentrated on the dynamics of the TIM-catalyzed reaction; but what of the enzyme's structure: why is TIM such an extremely good catalyst? What we know is (a) that there is an essential carboxylate group at the active site of the enzyme which may very well be the base responsible for proton shuttling between carbon centers;[27] (b) that the enediol intermediate is likely to be cis;[4] and (c) that there is probably an electrophilic catalyst that polarizes the substrate carbonyl group.[28] Further, the structure of the enzyme at high resolution has been solved;[7] this confirms the existence of the unique active-site glutamate in a pocket in the enzyme, which also contains histidine and lysine residues whose detailed function will presumably emerge when the structure of the enzyme–DHAP complex is completed.[7]

The idea that the enzyme base is a carboxylate group is attractive for a number of reasons. From the observation that there is some transfer of tritium from the 1*R* position of DHAP to the 2 position of GAP, it is likely that only one base on the enzyme is involved. It is conceivable that the proton transfer does occur by the handing on of the 1*R* proton from one enzyme base to another, in a "conducted tour" type of mechanism,[29] but the more economical view will be taken here. Possibly the bidentate nature of a carboxylate ion allows a basic oxygen atom to be in a position appropriate for proton abstraction whichever substrate is presented to the enzyme, without any time-consuming conformational change at the active site. A carboxyl group is also consistent with the fact that exchange of protons with solvent water is faster than transfer (oxygen–oxygen proton exchange being very rapid).[30]

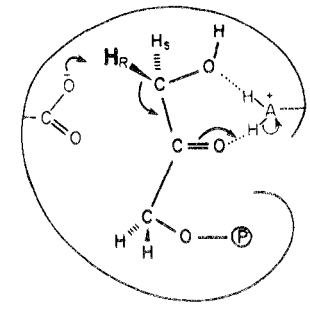

Figure 8. Putative formulation of the complex of dihydroxyacetone phosphate with triosephosphate isomerase. The carboxylate group of Glu_{165} abstracts the *pro-R* hydrogen, assisted by an electrophilic catalyst as shown.

The stereochemistry of the reaction also presents an interesting problem. On the reasonable basis that (a) there is only one base, and (b) a single base is most likely to shuttle protons between carbon centers on one side of the substrate, Rose showed that the stereochemistry of the labilized proton for a number of isomerases demanded a *cis*-enediol[4] (and, presumably, cisoid oxygens in each of the enzyme–substrate complexes as in Figure 2). It remains to be seen if the enzyme binds its substrates in a conformation satisfying the stereoelectronic requirement of periplanarity to a carbonyl π orbital[31] and, possibly, an appropriate orientation of lone-pair orbitals on the α-hydroxyl group analogous to the stereoelectronic effects described by Deslongchamps.[32]

The third feature of the catalysis is the likelihood of an electrophilic component in the catalysis. If the carbonyl group of dihydroxyacetone phosphate is reduced by tritiated borohydride in the presence of enzyme, stereoselective production of *sn*-[2-^{3}H]glycerol 3-phosphate occurs, showing that reduction on the enzyme surface occurs from the *si* face.[28] This is entirely consistent with the expectation that the enzyme blocks the *re* face since this is the face from which the proton is delivered to make the product D-GAP. The rate of reduction of enzyme-bound DHAP is nearly an

(24) It is interesting in this connection to speculate—as a number of people have—that many enzyme-catalyzed reactions will have two or more energy barriers of similar height. As long as one step in a reaction is much slower than all the others, speeding up that step speeds the whole reaction; but as soon as two barriers are of similar height, lowering of either one can have only a twofold effect on the overall rate, at most.

(25) We should not expect that this situation will be a rare one, and it is true for many enzymes that k_{cat}/K_m values are up in the range of 10^7–10^8 M^{-1} s^{-1}, which implies a very high catalytic effectiveness. Further, 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 (see W. W. Cleland, *Acc. Chem. Res.*, 8, 145 (1975)). It is evidently not uncommon that substrate-handling steps (i.e., "on" and "off" steps) are rate limiting, which requires that the actual catalytic steps for these enzymes are relatively fast. What is more significant now we can see the rates of all kinetically significant steps for an enzyme reaction (Figure 6) is that the erosion of the energetic hills and the filling in of the valleys has proceeded *just* to the state where further erosion is unprofitable. A suggestive sequence for the evolution of catalytic function is put forward by W. J. Albery and J. R. Knowles, *Biochemistry*, **15**, 5631 (1976).

(26) These arguments only apply, of course, to systems where the substrates and catalyst diffuse relatively freely in the cytoplasm or the relevant cytoplasmic compartment. While there have been various reports of the loose association of some glycolytic enzymes with each other and with subcellular particulate elements such as F actin [for references, see R. S. Foemmel, R. H. Gray, and I. A. Bernstein, *J. Biol. Chem.*, **250**, 1892 (1975); F. M. Clarke and C. J. Masters, *Biochim. Biophys. Acta*, **381**, 37 (1975); W. Tillman, A. Cordua, and W. Schroter, *ibid.*, **382**, 157 (1975); J. Mowbray and V. Moses, *Eur. J. Biochem.*, **66**, 25 (1976)], the importance of such association and compartmentalization in vivo is uncertain. In terms of glycolytic metabolism, a mixture of purified glycolytic enzymes is "almost identical" with intact muscle tissue [R. K. Scopes, *Biochem. J.*, **142**, 79 (1974)].

(27) F. C. Hartman, *Biochem. Biophys. Res. Commun.*, **33**, 888 (1968); **39**, 384 (1970); A. F. W. Coulson, J. R. Knowles, and R. E. Offord, *Chem. Commun.*, 7 (1970); F. C. Hartman, *Biochemistry*, **10**, 146 (1971); S. de la Mare, A. F. W. Coulson, J. R. Knowles, J. D. Priddle, and R. E. Offord, *Biochem. J.*, **129**, 321 (1972).

(28) M. R. Webb and J. R. Knowles, *Biochem. J*, **141**, 589 (1974); *Biochemistry*, **14**, 4692 (1975).

(29) D. J. Cram, F. Willey, H. P. Fischer, H. M. Relles, and D. A. Scott, *J. Am. Chem. Soc.*, **88**, 2759 (1966).

(30) Indeed, the exchange process shows no hydrogen isotope effect, which implies that the ionization of the conjugate acid of the active-site glutamic acid residue is not limited in rate by the ionization step itself but by some diffusive or solvent reorganization step subsequent to it: L. M. Fisher, W. J. Albery, and J. R. Knowles, *Biochemistry*, **15**, 5621 (1976).

(31) E. J. Corey and R. A. Sneen, *J. Am. Chem. Soc.*, **78**, 6269 (1956).

(32) P. Deslongchamps, C. Moreau, D. Frehel and P. Atlani, *Can. J. Chem.*, **50**, 3402 (1972); P. Deslongchamps, P. Atlani, D. Frehel and A. Malaval, *ibid.*, **50**, 3405 (1972); P. Deslongchamps, C. Lebreux, and R. Taillefer, *ibid.*, **51**, 1665 (1973); P. Deslongchamps, P. Atlani, D. Frehel, A. Malaval, and C. Moreau, *ibid.*, **52**, 3651 (1974).

order of magnitude faster than that of free DHAP (as is evident from the fact that, under conditions where less than 5% of the total DHAP is enzyme bound, the ratio of L- to D-glycerol phosphate [i.e., *sn*-glycerol 3-phosphate to *sn*-glycerol 1-phosphate] is 2:1[28]), which suggests that the substrate's carbonyl group is polarized on binding to the enzyme, facilitating enolization and (incidentally) accelerating the reduction by borohydride.

It appears, therefore, that the enolizations that constitute catalysis by TIM are effected by proton abstraction by a carboxylate base, which is accelerated by general acid catalysis[33] at the oxygen of the carbonyl group. Mechanistic economy (or the minimal number rule of Hanson and Rose[34]) would favor a formulation such as that shown in Figure 8.

(33) A. F. Hegarty and W. P. Jencks, *J. Am. Chem. Soc.*, 97, 7188 (1975).
(34) K. R. Hanson and I. A. Rose, *Acc. Chem. Res.*, 8, 1 (1975).

Envoi

It has proved possible to define the energetics of an enzyme-catalyzed reaction by using a combination of isotopic methods. It is clear from the free-energy profile that the enzyme has reached the end of its evolutionary development, the maximum flux of substrate being determined by a nonerodable diffusion limitation. The synthesis of this kinetic information with particularly promising structural studies (crystallographic,[7] spectroscopic, and chemical) should allow a more intimate description of an enzyme's catalytic act than has proved feasible hitherto.

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Electron Spin Resonance Studies of Erythrocyte Membranes in Muscular Dystrophy

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Background to the Various Disease States

Muscular dystrophy is a term which describes a class of diseases characterized by an inherited progressive muscle weakness and degeneration (dystrophy). Two common dystrophic conditions are Duchenne (DMD†) and myotonic (MyD) muscular dystrophy.[1] A third condition is not a dystrophic state; rather, congenital myotonia (CM) is a disease whose primary symptom is myotonia,[1] defined as a prolonged muscle contraction upon stimulation.

All three of these diseases are differentially inherited, and the clinical symptoms are expressed differently. DMD is inherited in males through a sex-linked recessive gene carried by the mother.[1] Dystrophy of proximal muscles (those near the torso) often appears in the first 5 years of life and rapidly progresses to complete immobilization in a relatively few years. In contrast, MyD, inherited as an autosomal (non-sex-linked) dominant trait by both males and females, initially demonstrates slowly progressive dystrophy of the distal musculature.[1] In addition to weakness, myotonia, and progressive dystrophy of striated muscle, many other symptoms are often present in this latter disease.[1] For example, malformation of cranial bones, testicular atrophy, presence of cataracts, abnormal electrocardiograms associated with cardiac symptoms, various endocrine abnormalities, and other symptoms are often observed.[1] CM is a much rarer disease and can be inherited as an autosomal recessive or autosomal dominant gene.[2]

Myotonia is measured electrically by electromyographic techniques.[1] When the electric output of the electrode in myotonic muscle is connected to an audio amplifier, a characteristic "dive-bomber" sound is heard due to the repetitive membrane depolarization. Should a myotonic patient exert a muscle he has not recently used, the muscle will remain involuntarily contracted for a longer time than would be the case for a person without myotonia. Myotonia in CM and MyD persists even in the presence of curare, an agent which blocks the electrochemical contact between nerve and muscle.[1] This fact suggests that the defect is localized to the muscle tissue itself. More recently, physiological experiments have localized the defect responsible for myotonia in these diseases to the muscle surface membrane.[3]

Much effort has been undertaken to define the primary biochemical alteration in each of these diseases.[4] No definite alterations in the proteins inti-

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† Abbreviations: DMD, Duchenne muscular distrophy; MyD, myotonic muscular distrophy; CM, congenital myotonia; ESR, electron spin resonance; 5-NS, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinyloxy (stearic acid 5-nitroxide or 5-NS); 5-NMS, 12-NMS, 16-NMS, methyl esters of 5-NS, 12-NS, or 16-NS; MAL-6, 2,2,6,6-tetramethylpiperidinyl-1-oxy-4-maleimide; ^{3}H-NEM, tritiated *N*-ethylmaleimide; DPH, diphenylhydantoin; RBC, red blood cell.

(1) R. D. Adams, D. Denny-Brown, and C. M. Pearson, "Diseases of Muscle", 2nd ed, Harper and Row, New York, N.Y., 1965.
(2) P. E. Becker in "New Developments in Electromyography and Clinical Neurophysiology", Vol. 1, J. E. Desmedt, Ed., Karger Press, Basel, 1973, pp 407–412.
(3) R. J. Lipicky and S. H. Bryant, in ref 2, pp 451–463.