The Mechanistic Pathway of a Mutant Triosephosphate Isomerase[a]

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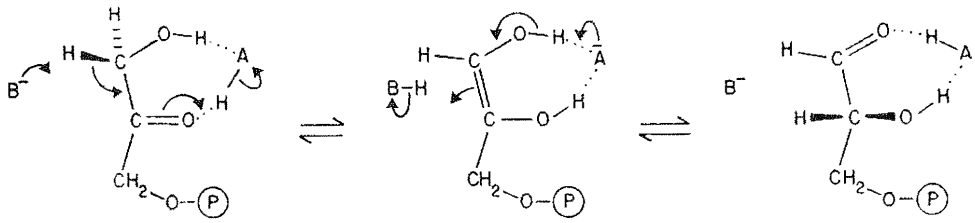
The glycolytic enzyme triosephosphate isomerase catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, following the pathway illustrated in SCHEME 1.[1,2] From a variety of kinetic,[3] stereochemical,[4] and chemical modification studies,[5,6] it is known that an enzymic base abstracts the 1-pro-*R* proton of dihydroxyacetone phosphate to yield the *cis*-enediol (or enediolate) intermediate, which then collapses as a proton is delivered to carbon-2 in the formation of D-glyceraldehyde 3-phosphate. When stereospecifically labeled 1-[(*R*)-^{3}H]- dihydroxyacetone phosphate is used as substrate, some 3% to 6% of the tritium label ends up at carbon 2 of the product D-glyceraldehyde 3-phosphate.[4] This small but significant level of proton transfer from carbon-1 to carbon-2 strongly suggests that there is a single base at the active site of the isomerase. The rate of exchange of the proton in B-H (SCHEME 1, at the enediol stage of the reaction) with the solvent is



SCHEME 1. Pathway of the reaction catalyzed by triosephosphate isomerase. *B* is an enzymic base (Glu-165), and *HA* is an enzymic acid (probably His-95 and/or Lys-13).

evidently very rapid, being about 20 times faster than the rate of collapse of the enediol to glyceraldehyde phosphate and loss of this product from the enzyme. Affinity labeling work with bromohydroxyacetone phosphate[6] and with glycidol phosphate[5] implicated a glutamic acid residue, Glu-165, as the single essential basic residue.

This conclusion was confirmed when the crystal structures of the triosephosphate isomerases from chicken muscle[7] and from yeast[8] were solved, and Glu-165 was found on one face of the active site cleft of the enzyme. The role of Glu-165 in the mechanism of the enzyme is secure. It is, moreover, chemically satisfying to have a bidentate base such as carboxylate at the active site of this enzyme, since one may presume that minimal motion will be required for the abstraction either of the 1-pro-*R* proton of dihydroxyacetone phosphate or of the carbon-2 proton of glyceraldehyde phosphate.

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The fact that the rate of the substrate interconversion mediated by triosephosphate isomerase is more than 10^9 times faster than that which would be observed by a nonenzymic carboxylate,[9,10] coupled with the existence of general acid as well as general base terms in the kinetics of simple enolizations,[11] prompted the search for an electrophilic component in the catalyzed reaction. Two kinds of experiment were performed.

First, we found that the rate of reduction of the carbonyl group of dihydroxyacetone phosphate by borohydride is about eight times faster when the substrate is bound to the enzyme than when it is free in solution.[12,13] Despite the expected slowing of the reduction due to the steric bulk of the protein, there is evidently a significant polarization of the carbonyl group when dihydroxyacetone phosphate is enzyme bound.

Such polarization was also indicated by the second kind of experiment in which the carbonyl stretching frequency of the free and the bound substrate was investigated by Fourier transform infrared spectroscopy.[14] We found that the carbonyl absorption of dihydroxyacetone phosphate is shifted by 19 cm^{-1} to lower frequencies on binding to the enzyme. This result is most readily interpreted in terms of carbonyl polarization by an enzymic electrophile at the active site of the isomerase. Such an electrophile would, of course, be expected to facilitate the enolization processes that constitute the covalency changes effected by the isomerase. Inspection of the crystal structures of the enzyme presently available does not allow the unambiguous identification of the putative catalytic electrophile(s), though two good candidates exist, His-95 and Lys-13, and it is likely that when the crystal structure of the enzyme:substrate complex is taken to higher resolution,[15] these questions will be resolved.

In an effort to evaluate the kinetic and mechanistic role of Glu-165, and to take a step towards defining the relationship between intermolecular distance and kinetic effect, we have used the methods of oligonucleotide-mediated site-specific mutagenesis to change Glu-165 to Asp. The chicken muscle enzyme (on which all our earlier mechanistic work was done) has been expressed from a cDNA clone in a strain of *E. coli* from which the endogenous bacterial isomerase has been deleted, and this enzyme has been subjected to the mutagenic alteration.[16] The three homogeneous enzymes, chicken enzyme from chicken, wild-type chicken enzyme from *E. coli*, and mutated (Glu-165 to Asp) chicken enzyme from *E. coli*, are shown in FIGURE 1. Kinetically, the wild-type chicken enzyme produced by *E. coli* is, as expected, indistinguishable from the enzyme isolated from chicken breast muscle. The mutant enzyme in which Glu-165 has been changed to Asp has, however, lost more than 99% of its catalytic activity.[16]

Two critical questions immediately arise. First, how do we know that the oligonucleotide-mediated change is the only one (*i.e.*, that the lower activity is due solely to the targeted alteration)? Second, how do we know that a protein having less than 1% of the catalytic activity of the native enzyme is not a mixture of 99% of inactive protein contaminated by 1% of the wild-type (arising either as a contaminant during protein purification, or as a genetic revertant during cell growth)? The first question has been answered by sequencing the whole of the mutated gene and demonstrating that the only differences are those intentionally created by the synthetic oligonucleotide.[16] The second question happens to be easy to answer in the present case, though this will certainly not be general. When a steady-state kinetic study was performed, the parameters shown in TABLE 1 were obtained.[16,17] It is evident that the values for K_m are somewhat different for the wild-type and mutant enzymes, and since (unlike V_{max}) K_m is a property only of the type of active enzyme molecule and not of the number of such molecules, the K_m difference means that the observed catalytic activity must derive from the mutant protein. It can also be seen from TABLE 1 that the Haldane relationship is obeyed, as it must be.

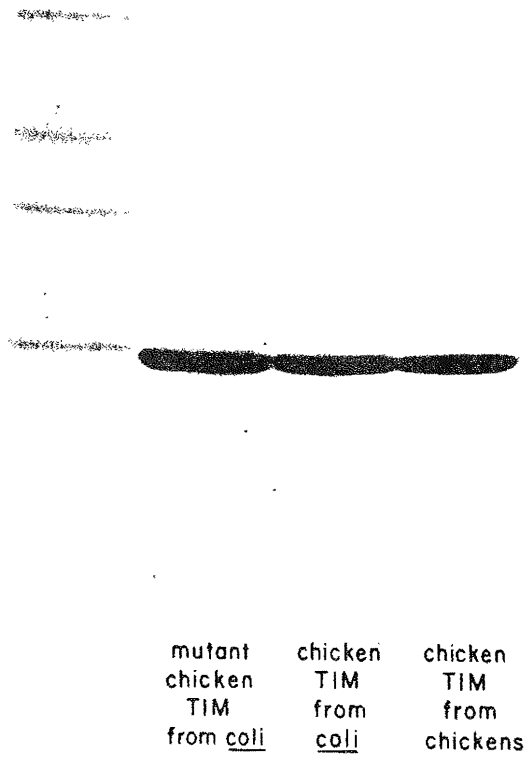

FIGURE 1. Polyacrylamide gel electrophoresis of triosephosphate isomerases under denaturing conditions.

Before deconvoluting the complex kinetic parameters k_{cat} and K_m and evaluating the kinetic effect of the Glu to Asp change at the level of the elementary step (to be reported elsewhere[18]), we must first ask if there has been a change in mechanism as a result of the alteration of the enzyme. We must be cautious of any large effect on catalytic activity, lest the pathway that the mutated enzyme follows is different from the wild-type. The most obvious concern in the present case is that whereas the Glu of the wild-type enzyme abstracts a substrate proton directly, the Asp of the mutant enzyme could act as a general base through an intervening water molecule (FIG. 2). We have approached this possibility in two ways. First, if there is an intervening water molecule in the Asp enzyme, we should expect a markedly larger solvent isotope effect since the two possible transition states involve different numbers of protons in flight. (The motion of protons could in principle be sequential, but would then involve some

TABLE 1. Steady-State Kinetic Parameters for the Wild-Type and Mutant Isomerases

	Dihydroxyacetone Phosphate as Substrate		D-Glyceraldehyde 3-Phosphate as Substrate		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	K_{eq}
Wild-type	430	0.97	4300	0.47	21
Mutant (Glu → Asp)	1.8	1.8	2.8	0.13	21

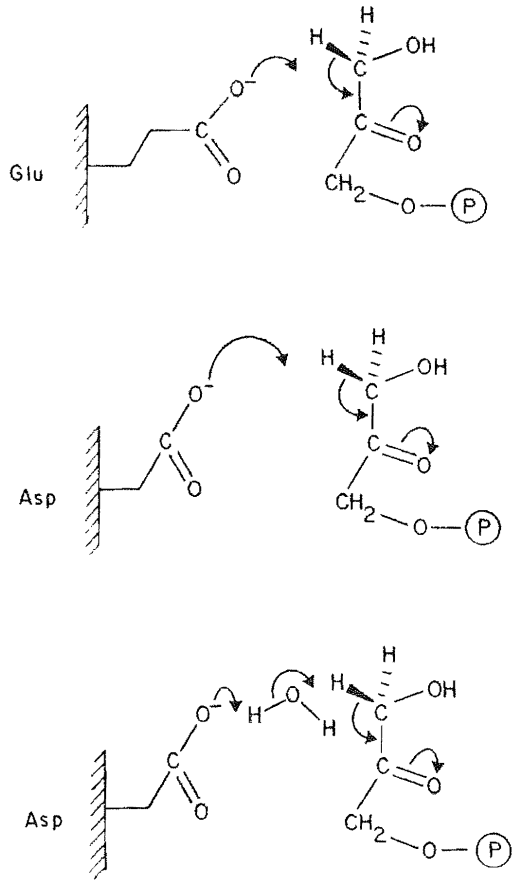

FIGURE 2. Illustration of the possible incursion of a water molecule between the substrate and the Asp residue of the mutant enzyme.

most improbable intermediate states.) In order both to probe a transition state for substrate enolization and to allow the same comparison for both wild-type and mutant enzymes, we determined the solvent isotope effect on V_{max} for D-glyceraldehyde 3-phosphate. These values are shown in TABLE 2. While there may well be effects of D_2O in the catalyzed reaction (*e.g.*, medium effects, subtle enzyme structural changes, effects on the electrophilic component, etc.) that we cannot define from the present results, a comparison of the values of V_{max} (H_2O)/V_{max} (D_2O) for mutant and wild-type is valid, and the data strongly suggest that these enzymes follow the same mechanistic course.

Second, if a water molecule were to interpose itself between the carboxylate of Asp-165 and carbon-1 of the substrate, we should expect that bromohydroxyacetone phosphate, which is a most effective affinity labeling reagent for Glu-165 of the

TABLE 2. Solvent Deuterium Isotope Effects for the Wild-Type and Mutant Isomerases with D-Glyceraldehyde 3-Phosphate as Substrate

	$V_{max}(H_2O)/V_{max}(D_2O)$
Wild-type enzyme	1.5 ± 0.1
Mutant enzyme	1.5 ± 0.2

wild-type enzyme,[6] would not inactivate the mutant. Indeed, the intervention of a water molecule could cause the mutant enzyme to catalyze the hydrolysis of the bromo compound. Neither of these expectations is realized. Bromohydroxyacetone phosphate inactivates the mutant enzyme stoichiometrically (see FIGURE 3) and there is no production of the hydrolysis product, dihydroxyacetone phosphate. It appears, then, that the mechanistic path followed by the mutant enzyme is unchanged.

The next problem, which must concern any investigation of mutant enzymes the catalytic activity of which are different from the wild-type, is whether the mutation has merely shifted the pH dependence of the catalyzed reaction.[19,20] This is not a major concern in the present case, since the intrinsic pK_a values for the side chain carboxyl groups of Glu and Asp residues differ by only about 0.4 unit,[21] and such a small shift in the pH-activity curve could not cause the observed 10^2- to 10^3-fold change in catalytic activity. Nevertheless, this point has been checked experimentally, and it is clear from the results shown in FIGURE 4 that the two enzymes have very similar pH dependencies, so the observed kinetic differences between wild-type and mutant enzymes do not derive from differences in the ionization behavior of catalytic groups.

Finally, we may ask whether the mutation has primarily affected substrate recognition or the act of catalysis. For most enzymes (and the isomerase is no exception), K_m and k_{cat} are nontrivial combinations of the rate constants for elementary steps, and it is dangerous to take the simplistic view that K_m measures substrate binding and k_{cat} measures the rate of a particular step in the subsequent catalytic reaction. Ideally, the kinetic behavior of a mutant enzyme must be completely analyzed, and such an analysis for the present case will be presented elsewhere.[18] Suffice it here to say that the alteration of Glu-165 to Asp appears *not* significantly to perturb the substrate recognition and binding interactions of the isomerase. We have investigated the binding of the so-called transition state analogue, phosphoglycolohydroxamate,[22] and found that the inhibition of the wild-type and mutant enzymes is comparable. The K_i values are 7 μM (wild-type) and 20 μM (mutant). This difference is very small when compared with the 10^2- to 10^3-fold difference in k_{cat} values (TABLE 1), and gives us some confidence that the reduction in catalytic activity does not reflect

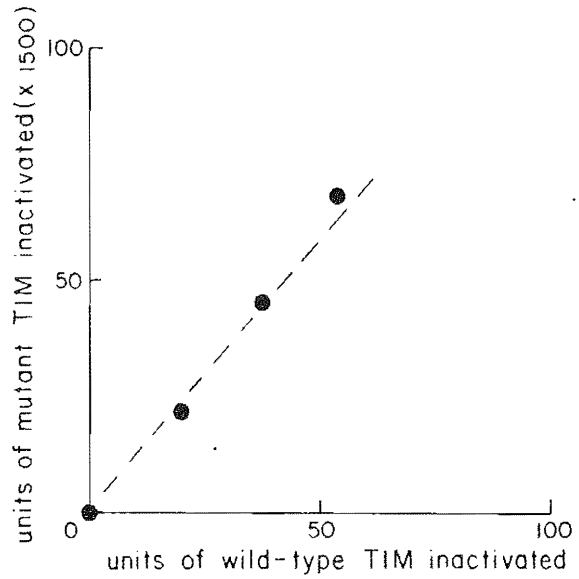

FIGURE 3. Plot of the inactivation of wild-type (abscissa) and mutant (ordinate) isomerases by increasing amounts of the active-site directed reagent, bromohydroxyacetone phosphate. The values for the mutant enzyme are multiplied by 1500 (the ratio of the k_{cat} values).

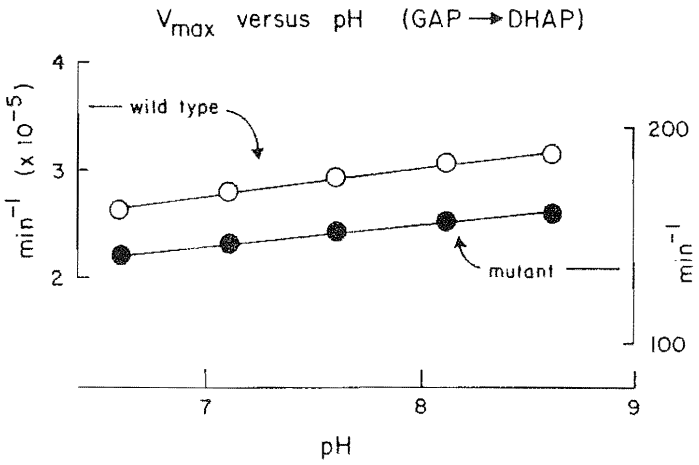

FIGURE 4. The pH variation of V_{max} for D-glyceraldehyde 3-phosphate, for both wild-type and mutant enzymes.

a major change in the structure of the protein. While any more definitive statement must await a crystallographic difference map at high resolution, the data presented here suggest that we shall, in the fullness of time, be able precisely to correlate the structural change with the kinetic behavior of the mutant enzyme.

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Rhodopsin, the Visual Pigment, and Bacteriorhodopsin[a]

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I have two proteins in my title. Rhodopsin belongs to the large field of visual sensory perception in vertebrates and invertebrates. Bacteriorhodopsin (BR), a more recently discovered light-transducing protein, is found in certain archae-bacteria, a class of bacteria believed to be very primitive. Light transduction by bacteriorhodopsin results in the production of metabolic energy through the synthesis of ATP. An important and remarkable similarity the two proteins share is in the molecular nature of the light-absorbing chromophore. In both cases it is retinal aldehyde, linked to the amino group of a lysine residue that seems to be embedded in the membrane. I shall first discuss briefly rhodopsin, about which we know even less than bacteriorhodopsin, and then review the work on the latter, which has proved to be an attractive system for the study of integral membrane proteins.

RHODOPSIN

Rhodopsin is the major membrane protein in the discs of the outer segments of rod cells in the vertebrate retina (see FIGURE 1 for a schematic presentation of the functional organelles of the rod cell). We are beginning to obtain glimpses of the chemistry and biochemistry that is initiated following the capture of a photon by rhodopsin, the photoreceptor protein. An outline of the cascade of reactions, not currently well characterized, is shown in FIGURE 2 (courtesy of Lubert Stryer). The overriding biological principle in this and other sensory systems must be transduction and amplification of the signal received, in this case following light transduction. The photon absorption by rhodopsin must be presumed to bring about "activation" through a conformational change. Bleached rhodopsin then can interact with the GDP-bound form of GTPase and converts it to an activated form (FIG. 2) which can in turn activate a phosphodiesterase that can hydrolyze or modulate the concentration of cyclic guanosine 3',5'-cyclic phosphate. The latter seems to be a transmitter or second messenger that regulates the states of the ion conductance channel in the plasma membrane. The result is the hyperpolarization of the rod cell and the activation of the synapse that transmits the signal to the series of cells in succession (FIG. 3, from John Dowling) and ultimately to the optic nerve and the brain.[1]

George Wald, who pioneered biochemical studies of vision, discovered several intermediates with characteristic spectroscopic properties in the photochemistry of rhodopsin. A modern version of these photochemical intermediates is shown in FIGURE

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