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Reaction Energetics of a Mutant Triosephosphate Isomerase in Which the Active-Site Glutamate Has Been Changed to Aspartate[†]

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Received April 14, 1986; Revised Manuscript Received July 1, 1986

ABSTRACT: The essential catalytic base at the active site of the glycolytic enzyme triosephosphate isomerase is the carboxylate group of Glu-165, which directly abstracts either the 1-*pro-R* proton of dihydroxyacetone phosphate or the 2-proton of (*R*)-glyceraldehyde 3-phosphate to yield the *cis*-enediol intermediate. Using the methods of site-directed mutagenesis, we have replaced Glu-165 by Asp. The three enzymes chicken isomerase from chicken muscle, wild-type chicken isomerase expressed in *Escherichia coli*, and mutant (Glu-165 to Asp) chicken isomerase expressed in *E. coli* have each been purified to homogeneity. The specific catalytic activities of the two wild-type isomerases are identical, while the specific activity of the mutant enzyme is reduced by a factor of about 1000. The observed kinetic differences do not derive from a change in mechanism in which the aspartate of the mutant enzyme acts as a general base through an intervening water molecule, because the D₂O solvent isotope effects and the stoichiometries of inactivation with bromohydroxyacetone phosphate are identical for the wild-type and mutant enzymes. Using the range of isotopic experiments that were used to delineate the free-energy profile of the wild-type chicken enzyme, we here derive the complete energetics of the reaction catalyzed by the mutant protein. Comparison of the reaction energetics for the wild-type and mutant isomerases shows that only the free energies of the transition states for the two enolization steps have been seriously affected. Each of the proton abstraction steps is about 1000-fold slower in the mutant enzyme. Evidently, the excision of a methylene group from the side chain of the essential glutamate has little effect on the free energies of the intermediate states but dramatically reduces the stabilities of the transition states for the chemical steps in the catalyzed reaction.

The opportunity to tinker with the amino acid sequence of enzymes by using the methods of site-directed mutagenesis is proving to be irresistible. Yet to avoid the aimless and the

uninterpretable, the enzymologist must be prepared to analyze both the structural and functional consequences of an amino acid alteration in detail. The most informative first steps will therefore be taken with enzymes that are already well characterized in structural, mechanistic, and energetic terms. We report here the effects on the reaction energetics of a single change at the active site of such an enzyme, triosephosphate isomerase.

The three-dimensional structure of native triosephosphate isomerase is known to atomic resolution. Additionally, since the isomerase catalyzes the interconversion of one substrate and one product, the structure of a productive complex of the

[†] This work was supported by the National Science Foundation, the National Institutes of Health, and Merck Sharp & Dohme. E.L.S. held a National Science Foundation Graduate Fellowship.

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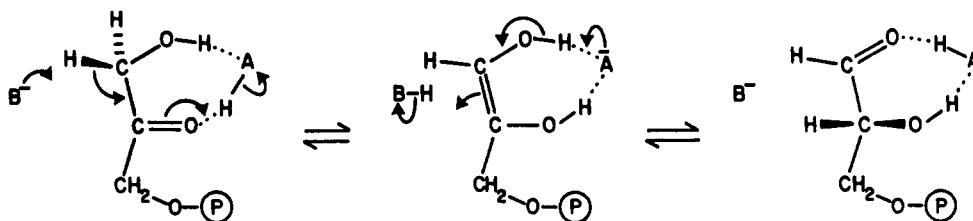


FIGURE 1: Reaction catalyzed by wild-type triosephosphate isomerase. B^- is the enzymic base (Glu-165), and HA is an enzymic acid (Lys-13, or His-95, or both).

enzyme with its natural substrate can be studied. Thus, the crystal structure of triosephosphate isomerase from chicken muscle has been determined to 2.5 Å (Banner et al., 1975), and the complex of this enzyme with substrate has been studied at 6.0 Å (Phillips et al., 1977). Further, the structure of the isomerase from yeast has been determined to 1.9 Å (Alber et al., 1986), and the complexes of this enzyme with substrate (Alber et al., 1981) and with the competitive inhibitor phosphoglycolohydroxamate (Davenport, 1986) have been studied at 3.5 and 1.6 Å, respectively. Other details of the interaction between the enzyme and its substrates are available from nuclear magnetic resonance spectroscopy (Browne et al., 1976; Webb et al., 1977) and from Fourier-transform infrared spectroscopy (Belasco & Knowles, 1980). There is no doubt that any mutant isomerase that we may generate can be well characterized in structural terms.

Early mechanistic work (Rieder & Rose, 1959; Rose, 1962) demonstrated that the reaction catalyzed by triosephosphate isomerase follows the pathway shown in Figure 1. An enzymic base abstracts either the 1-*pro-R* proton of dihydroxyacetone 3-phosphate¹ or the 2-proton of (*R*)-glyceraldehyde 3-phosphate to yield a *cis*-enediol (or enediolate) intermediate. When stereospecifically labeled [1(*R*)-³H]dihydroxyacetone 3-phosphate is used as substrate, between 3 and 6% of the tritium label appears on C-2 of the product glyceraldehyde phosphate, the actual amount of transfer depending on the extent of the reaction (Herlihy et al., 1976). This small but significant amount of proton transfer from C-1 to C-2 suggests that a single active site base abstracts the proton from each substrate. Affinity labeling work with glycidol phosphate (Waley et al., 1970) and with bromohydroxyacetone phosphate (de la Mare et al., 1972) implicated Glu-165 as the essential basic residue, and the crystal structures of isomerase and the isomerase-substrate complex have confirmed this view. The role of Glu-165 in the mechanism of the wild-type enzyme is secure.

Catalysis by triosephosphate isomerase also has an electrophilic component. The participation of acidic residues in the catalytic mechanism is not surprising. The rate of substrate interconversion mediated by the enzyme is more than 10⁹ times faster than that by acetate ion (Hall & Knowles, 1975; Richard, 1984), and simple enolization reactions are known to be catalyzed by general acids as well as by general bases (Hegarty & Jencks, 1975). Polarization of the substrate carbonyl group (presumably by an active site electrophile) has been detected by two types of experiments. First, the rate of reduction of the carbonyl group of dihydroxyacetone phosphate by borohydride is about 8 times faster when the substrate is bound to the enzyme than when the substrate is free in solution (Webb & Knowles, 1974, 1975). Second, the carbonyl

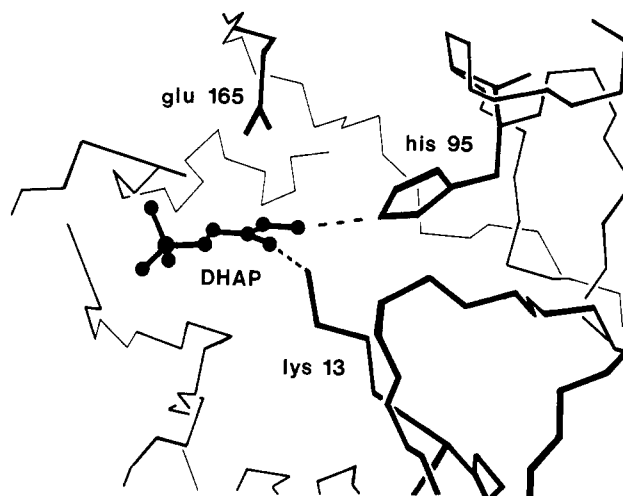


FIGURE 2: Active site of wild-type triosephosphate isomerase. The coordinates are those of the native chicken enzyme (Banner et al., 1976). Dihydroxyacetone phosphate is in the extended cisoid conformation (Rose, 1962) and has been positioned to minimize non-bonding contacts between the enzyme and the substrate. The orientation of dihydroxyacetone phosphate with respect to the enzyme is in accord with the abstraction of the 1-*pro-R* proton of the substrate by Glu-165, the polarization of the substrate carbonyl group by His-95, and the hydrogen bonding of the substrate hydroxyl group to Lys-13. The movement of the loop of residues 168–177 that is evident from the structure of the enzyme–substrate complex (Phillips et al., 1977; Alber et al., 1981) is not shown.

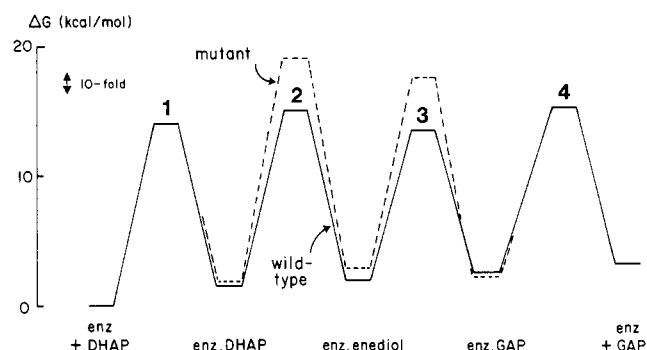


FIGURE 3: Free-energy profiles for reaction catalyzed by wild-type and mutant isomerases. The wild-type profile is from Alber and Knowles (1976b).

stretching frequency of dihydroxyacetone phosphate decreases by 19 cm⁻¹ on binding to the enzyme (Belasco & Knowles, 1980). Although the available crystal structures of the enzyme do not allow the unambiguous identification of the putative electrophilic residue(s), Lys-13 and His-95 are good candidates. The outlines of push-pull catalysis for the two enolization steps seem evident from the structure of the active site shown in Figure 2.

The rates of the individual steps in the reaction catalyzed by triosephosphate isomerase catalysis have been determined (Albery & Knowles, 1976b) and are presented as a free-energy profile in Figure 3 (Knowles & Albery, 1977). The particular

¹ Nomenclature: dihydroxyacetone phosphate, dihydroxyacetone 3-phosphate; glyceraldehyde phosphate, (*R*)-glyceraldehyde 3-phosphate (otherwise D-glyceraldehyde 3-phosphate); glycerol phosphate, (*R*)-glycerol phosphate (otherwise *sn*-glycerol 3-phosphate); phosphoglycerate, 3-phospho-(*R*)-glycerate (otherwise 3-phospho-D-glycerate).

ground state and transition state that define the largest free-energy difference in the profile for the wild-type enzyme illustrate the efficiency of catalysis by the enzyme. Specifically, the highest transition state for the wild-type enzyme is that for the binding of glyceraldehyde phosphate. The absolute magnitude of the rate constant for this step ($4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) together with viscosity variation experiments (R. T. Raines, S. C. Blacklow, and W. A. Lim, unpublished work) indicate that this transition state relates to a diffusive process. The lowest ground state in the free-energy profile (at the ambient substrate levels in vivo) is not that of a liganded enzyme form but is that of unliganded enzyme plus dihydroxyacetone phosphate. Since the relative free energies of the highest transition state and the lowest ground state in the free-energy profile for the wild-type enzyme are independent of the catalytic power of the enzyme, this largest free-energy difference cannot be reduced by the catalyst. Wild-type triosephosphate isomerase is therefore "perfect" in the sense that any further acceleration of the catalytic steps (as distinct from the "on-off" steps) would have no effect on the rate of the overall reaction (Albery & Knowles, 1976c, 1977; Knowles & Albery, 1977).

The above summary of the structure, mechanism, and energetics of triosephosphate isomerase suggests that this enzyme is a splendid candidate for site-directed mutagenesis. The opportunity exists for a thorough evaluation of the consequences of subtle changes in critical amino acid residues, in the hope that we may better understand the origins and nature of the dramatic catalysis mediated by this enzyme. We have begun this process by making a small change in one of the enzymic residues that directly interacts with the substrate. By changing Glu-165 into Asp, we have retained the carboxyl group but (presumably) moved it away from the substrate. Our specific goal in this instance is to gain some insight into the relationship between distance and reaction rate. Although the precise structural consequences of the change from Glu to Asp must await high-resolution crystallographic data on the mutant isomerase-substrate complex, we here report the effects on the reaction energetics of moving the essential active site base by something less than 1 Å.

EXPERIMENTAL PROCEDURES

Materials. *Escherichia coli* strains DF502(pX1) (Straus & Gilbert, 1985) and DF502(ptm2) (Straus et al., 1985) were the generous gifts of D. R. Straus. Plasmid pX1 carries the β -lactamase gene and a cDNA copy of the gene for triosephosphate isomerase from chicken breast muscle under the control of a *trc* promoter. Plasmid ptm2 was derived from plasmid pX1 by using a synthetic oligonucleotide encoding Asp in place of Glu-165 in the chicken isomerase protein (Straus et al., 1985). Each plasmid was expressed in DF502, a streptomycin-resistant *E. coli* strain from which the endogenous *E. coli* isomerase gene has been deleted.

Wild-type triosephosphate isomerase (chicken breast muscle) was prepared by J. G. Belasco according to Putman et al. (1972) and McVittie et al. (1972). Aldolase (rabbit muscle), alkaline phosphatase (bovine intestine), enolase (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), α -glycerophosphate dehydrogenase (rabbit muscle), 3-phosphoglycerate kinase (yeast), and phosphoglycerate mutase (rabbit muscle) were obtained as crystalline suspensions in ammonium sulfate from Sigma Chemical Co. (St. Louis, MO). Each dehydrogenase was treated with bromohydroxyacetone phosphate to remove triosephosphate isomerase activity (de la Mare et al., 1972) and then dialyzed exhaustively against 0.1 M triethanolamine hydrochloride

buffer, pH 7.6, containing EDTA² (20 mM) and 2-mercaptoethanol [0.01% (v/v)].

Dihydroxyacetone phosphate was synthesized from 3-chloro-1,2-propanediol by the method of Ballou (1960). Bromohydroxyacetone phosphate was synthesized according to de la Mare et al. (1972). Phosphoglycolohydroxamate (dicyclohexylammonium salt) was synthesized by J. G. Belasco as reported in Belasco and Knowles (1980). [$1(R)^{-3}H$]Dihydroxyacetone 3-phosphate ($10.6 \pm 0.1 \mu\text{Ci}/\mu\text{mol}$) was prepared according to Herlihy et al. (1976). (*RS*)-Glyceraldehyde 3-phosphate (diethyl acetal, monobarium salt), 2,3-diphospho-(*R*)-glycerate (pentacyclohexylammonium salt), NAD⁺, NADH (disodium salt), ATP (disodium salt) Dowex 50 W (H⁺ form, 100–200 mesh, 4% cross-linked), Dowex 1 (Cl[−] form, 200–400 mesh, 8% cross-linked), and Sephadex G-100 (20–50- μm bead size) were from Sigma. QAE-Sephadex A-50 (Cl[−] form) was from Pharmacia Fine Chemicals (Piscataway, NJ). DEAE-cellulose was from Whatman, Inc. (Clifton, NJ). D₂O (99.8%) was from Merck & Co. (Rahway, NJ). Bacto tryptone and Bacto yeast extract were from Difco Laboratories (Detroit, MI). Ammonium sulfate (enzyme grade) was from Schwarz/Mann (Cambridge, MA). Toluene (scintillation grade) was from Fisher (Medford, MA). Bio-Solv 3 solubilizer was from Beckman (Fullerton, CA). Liquefluor scintillator was from New England Nuclear (Boston, MA). Tritiated water (5 Ci/mL) was from Amersham (Chicago, IL). Dioxane was distilled over sodium benzo-phenone before use. All other chemicals and solvents were the best grades available commercially.

Methods. Rich medium is supplemented medium YT (Miller, 1972) and contains (in 1 L) Bacto tryptone (8 g), Bacto yeast extract (5 g), NaCl (5 g), dextrose (1 g), streptomycin sulfate (100 mg), and ampicillin (sodium salt) (100 mg). All media were prepared in distilled, deionized water.

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate [0.1% (w/v)] according to Laemmli (1970) and Laemmli and Favre (1973). The stacking gel was buffered with 0.5 M Tris-HCl at pH 6.8, and the separating gel [containing 12% (w/v) acrylamide] was buffered with 1.5 M Tris-HCl at pH 8.8. Typically, 25 μg of protein was loaded per lane. Gels were fixed with aqueous methanol [50% (v/v)] and visualized with Coomassie Brilliant Blue. The molecular weight standards (Sigma) were α -lactalbumin (14 200), trypsinogen (24 000), carbonic anhydrase (29 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), egg albumin (45 000), and bovine serum albumin (66 000).

Samples (200 μL) for radiochemical analysis were dissolved in scintillation cocktail (12 mL) and counted in a Beckman LS233 or a Beckman LS1801 automatic liquid scintillation counter. The scintillation cocktail contained toluene [85% (v/v)], Bio-Solv 3 solubilizer [10% (v/v)], and Liquefluor scintillator [5% (v/v)]. pH was measured with a Radiometer RHM62 pH meter fitted with a Radiometer GK2320 electrode, calibrated with Fisher standard buffer solutions. Conductivity was determined with a Radiometer CDM3 conductivity meter. Ultraviolet absorbance was measured with a Perkin-Elmer 554 spectrophotometer. An extinction coefficient for NADH of $6220 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm was assumed

² Abbreviations: ATP, adenosine 5'-triphosphate; DEAE-cellulose, diethylaminoethylcellulose; DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetate; GAP, glyceraldehyde phosphate; NAD⁺, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; QAE, [diethyl(2-hydroxypropyl)-amino]ethyl; TIM, triosephosphate isomerase; Tris, tris(hydroxymethyl)aminomethane.

(Horecker & Kornberg, 1948). Computer programs were written in Microsoft Basic and run on an Apple Macintosh computer.

Protein solutions were concentrated with Amicon PM-10 Diaflo ultrafiltration membranes (M_r 10 000 cutoff). Fisher dialysis tubing (M_r 12 000–14 000 cutoff) was pretreated according to Brewer (1974). The concentration of protein in heterogeneous solutions was determined according to Bradford (1976). The concentration of isomerase in homogeneous solutions was calculated from the A_{280} by assuming that the $A_{280}^{0.1\%}$ is 1.21 for a 10-mm light path (Miller & Waley, 1971; Furth et al., 1974). Triosephosphate isomerase was assayed at pH 7.6 by the method of Plaut and Knowles (1972). A subunit M_r of 26 500 was assumed (Putman et al., 1972; Banner et al., 1975), and kinetic parameters quoted are for a single subunit. Protein sequences were determined by automated Edman degradation with an Applied Biosystems 470A sequencer. Cleaved residues were identified with a Hewlett-Packard 1090 high-performance liquid chromatograph equipped with a 1040A diode array detector. About 1 nmol of isomerase subunit was used in each sequence analysis. All enzyme-catalyzed reactions were run at 30 °C. All other experimental manipulations were done at 4 °C unless otherwise noted.

Mutant Isomerase. *E. coli* strain DF502(ptm2) was grown from a single colony in rich medium (100 mL). This growth was used to inoculate a fermenter containing rich medium (90 L), and gentle oxygen aeration at 37 °C for 45 h afforded 360 g of wet cell paste. The cells were resuspended in H₂O (350 mL), and crude cell lysates were prepared by passing the cell suspension through a Manton-Gaulin mill 3 times at 8000 psi. Cell debris was removed by centrifugation at 165 000g for 1 h. Ammonium sulfate (548 g) was added slowly to the stirred supernatant (1255 mL). The suspension was stirred gently for 2 h, and the precipitate was then removed by centrifugation at 120 000g for 1 h. Further ammonium sulfate (174 g) was then added slowly to the stirred supernatant (1300 mL). The suspension was stirred gently for 2 h, and the precipitate was collected by centrifugation at 120 000g for 4 h. The pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.3 (40 mL), and the resulting solution was dialyzed exhaustively against the same buffer. The dialyzed solution (conductivity 2 mS) was loaded onto a column (24 cm × 20 cm²) of QAE-Sephadex A-50 that had been equilibrated with 10 mM Tris-HCl buffer, pH 7.3. The column was eluted at a rate of 2 mL/min with the same buffer, and fractions (18 mL) were collected. The fractions containing isomerase activity were analyzed by polyacrylamide gel electrophoresis, and those fractions having no contaminant of 40 000 < M_r < 60 000 (fractions 21–30) were pooled and concentrated. The resulting solution (3 mL) was loaded onto a column (87 cm × 5.3 cm²) of Sephadex G-100 that had been equilibrated with 50 mM Tris-HCl buffer, pH 7.3. The column was eluted at a rate of 8 mL/h with the same buffer, and fractions (5 mL) were collected. The fractions containing isomerase activity (fractions 58–70) were homogeneous by polyacrylamide gel electrophoresis and were pooled and concentrated.

Wild-Type Isomerase from *E. coli*. Wild-type chicken triosephosphate isomerase was purified from *E. coli* DF502(pX1) in a procedure analogous to that described above with two exceptions. First, the fermenter growth yielded 500 g of wet cell paste, only a portion of which (130 g) was used in the enzyme purification. Second, the loaded QAE-Sephadex A-50 column was washed with 10 mM Tris-HCl, pH 7.8, at a rate of 2 mL/min, and the column was eluted at a rate of

2 mL/min with a linear gradient (1 L + 1 L) of Tris-HCl (10–150 mM), pH 7.8.

pH Dependence of k_{cat} . The 0.1 M triethanolamine hydrochloride buffers were prepared with pH values of 6.6, 7.1, 7.6, 8.1, and 8.6. No EDTA was added to the buffers, and no attempt was made to keep the ionic strength constant in the different buffers. Wild-type (25 µL of a solution of 0.24 µg/mL) and mutant (5 µL of a solution of 2.5 mg/mL) isomerase were assayed at each pH with glyceraldehyde phosphate (2.0 mM). The k_{cat} values were calculated by averaging the reaction velocity per isomerase subunit from duplicate assays and correcting this value for the subsaturating concentration of glyceraldehyde phosphate with the appropriate K_m value for the wild-type (Putman et al., 1972) or mutant (Straus et al., 1985) isomerase.

Stereochemical Integrity. (A) Glyceraldehyde Phosphate as Substrate. The concentration of (*R*)-glyceraldehyde phosphate in a stock solution of the racemic material was measured in triplicate with the usual coupled assay of wild-type triosephosphate isomerase/glycerolphosphate dehydrogenase/NADH (Plaut & Knowles, 1972). The concentration of substrate in the racemic mixture available to the mutant isomerase was then measured under the same conditions. When the change in absorbance with time was zero, wild-type isomerase was added to assay for the presence of any unreacted (*R*)-glyceraldehyde phosphate.

(B) Dihydroxyacetone Phosphate as Substrate. [1(*R*)-³H]dihydroxyacetone 3-phosphate was prepared by incubation of unlabeled material (14 µmol) with ³H₂O, (26 mCi, 0.5 mL) in the presence of either wild-type or mutant isomerase (0.16–0.32 unit) for 20 h at 25 °C. The solvent was removed by lyophilization, and the triosephosphates were purified by chromatography on a column of Dowex 1 (Cl[−] form) as described by Leadlay et al. (1976), except that the small amount of (*R*)-glyceraldehyde phosphate (approximately 3%) was not removed from the combined triosephosphates by aldolase treatment. A portion (1 µmol) of each of these samples was then treated with wild-type isomerase (38 unit) in ¹H₂O (1 mL) for 3 h at 25 °C. A portion (400 µL, 10⁵ dpm) was removed, and the ³H₂O isolated by bulb-to-bulb distillation in a closed, evacuated apparatus. The residue was redissolved in ¹H₂O (400 µL), and the distillation was repeated. Samples (50 µL) of the combined distillates, and of the redissolved residue, were taken for scintillation counting. The percentage of the tritium label in each sample that was labilized from the [³H]dihydroxyacetone phosphate by the wild-type isomerase was then calculated.

D₂O Solvent Isotope Effect on k_{cat} for Glyceraldehyde Phosphate. Deuterated buffer solution was prepared as follows. The 0.1 M triethanolamine hydrochloride buffer, pH 7.6 (50 mL), containing EDTA (5 mM), was evaporated to dryness under reduced pressure, and the residue was dissolved in D₂O (5 mL). The resulting solution was again reduced to dryness, and the residue was dissolved in D₂O (40 mL). The pD of this solution was adjusted to 8.1 (pH meter reading 7.7) with 38% DCl in D₂O, and the volume was then adjusted to 50 mL by the addition of D₂O. [The dissociation of nitrogen and oxygen bases will be equivalent in D₂O and H₂O when pD = pH + 0.5 (Schowen, 1977).] The wild-type and mutant isomerases were assayed at seven different concentrations of glyceraldehyde phosphate in H₂O or D₂O buffer. The solutions of glyceraldehyde phosphate and of NADH used in the assay in D₂O buffer were made in D₂O. The stability of the isomerases in D₂O was checked by assaying each isomerase before and 72 h after incubation at 20 °C in deuterated buffer.

Titration with Bromohydroxyacetone Phosphate. Stock ethereal bromohydroxyacetone phosphate (~20 mM) was diluted with dioxane to a concentration of ~5 μ M. Aqueous solutions (100 μ L) containing 0.1 M triethanolamine hydrochloride buffer, pH 7.6, EDTA (20 mM), and isomerase (either wild-type or mutant enzyme at 13 μ g/mL) were mixed with dioxane solutions (30 μ L) containing varying amounts of bromohydroxyacetone phosphate. The resulting solutions were incubated for 1 h at 20 °C and then assayed for isomerase activity.

Inhibition by Phosphoglycolohydroxamate. The concentration of a solution of phosphoglycolohydroxamate was determined by colorimetric assay (Ames, 1966) of the inorganic phosphate released by alkaline phosphatase. Wild-type or mutant isomerase was assayed at five different concentrations of glyceraldehyde phosphate in the presence of phosphoglycolohydroxamate (10 mM). The V_{\max} and K_m were calculated with the nonlinear least-squares computer program HYPERO (Cleland, 1979). The dissociation constant for the enzyme-inhibitor complex, K_i , was calculated as described by Segel (1975).

Fate of Tritium Label of [1(R)-³H]Dihydroxyacetone 3-Phosphate. An isomerase-catalyzed reaction was run as described in Herlihy et al. (1976) except that the reaction was initiated by the addition of mutant isomerase. Tritiated water was removed by lyophilizing the quenched reaction mixture, then dissolving the resulting residue in 0.16 mM HCl (2 mL), and then lyophilizing this solution. Isomerase and glyceraldehyde-3-phosphate dehydrogenase were removed by dissolving the residue in 0.16 mM HCl (2 mL) and passing the resulting solution through a column (5 cm \times 0.25 cm²) of Dowex 50 W (H⁺ form). Dihydroxyacetone phosphate was converted to the more stable glycerol phosphate as follows. The pH of the eluate (~10 mL) from the Dowex 50 W column was raised to 7.0 by the careful addition of NaOH (0.1 M). The resulting solution was quickly evaporated under reduced pressure, and 0.1 M triethanolamine hydrochloride buffer, pH 7.6 (2.5 mL), containing EDTA (20 mM) and NADH (50 mL of a solution of 14 mg/mL), was added to the residue. The absorbance at 340 nm was recorded before and 30 min after the addition of α -glycerophosphate dehydrogenase (10 μ L of a solution of 12 mg/mL). The yield of glycerol phosphate was typically about 90%.

Glycerol phosphate was separated from phosphoglycerate as follows. The solution containing glycerol phosphate and phosphoglycerate was diluted with 5 mM triethanolamine hydrochloride buffer, pH 7.1 (80 mL), and this solution (conductivity <1 mS) was applied to a column (13 cm \times 1.7 cm²) of DEAE-cellulose that had been equilibrated with the same buffer. The column was washed with the same buffer (40 mL) and then eluted with a linear gradient (80 mL + 80 mL) of triethanolamine hydrochloride (5–300 mM), pH 7.1. Fractions (3 mL) were collected, and the specific radioactivity of glycerol phosphate and phosphoglycerate in each fraction was determined. To check on the location of the ³H label in the phospho[³H]glycerate, the hydrogen on C-2 was washed into the solvent by treatment with phosphoglycerate mutase and enolase (Dinovo & Boyer, 1971). This check was performed as described in Herlihy et al. (1976).

Appearance of Solvent Tritium in Substrate Dihydroxyacetone Phosphate and in Product. An isomerase-catalyzed reaction was run as described in Maister et al. (1976), except that the reaction was initiated by the addition of mutant isomerase. Removal of tritiated water and enzymes, conversion of dihydroxyacetone phosphate to the more stable glycerol

Table I: Purification of Mutant Triosephosphate Isomerase from *E. coli* Strain DF502(ptm2)

step	total units	sp act. (units/mg)	x-fold purification	yield (%)
crude lysate	1240	0.0415	1	100
ammonium sulfate pellet	614	0.345	8	50
QAE-Sephadex	201	6.66	160	16
Sephadex G-100	115	7.28	175	9.3

phosphate, and separation of glycerol phosphate from phosphoglycerate were all done as described above.

Appearance of Solvent Tritium in Substrate Glyceraldehyde Phosphate and in Product. An isomerase-catalyzed reaction was run as described in Fletcher et al. (1976), except that the reaction was initiated by the addition of mutant triosephosphate isomerase, and the extent of reaction was monitored by the absorbance at 340 nm in a 2-mm light-path optical cuvette. Tritiated water, isomerase, and α -glycerophosphate dehydrogenase (in place in glyceraldehyde-3-phosphate dehydrogenase) were removed as described above. Glyceraldehyde phosphate was converted to the more stable phosphoglycerate as follows. The pH of the eluate from the Dowex 50 W column was raised to 7.0 by the careful addition of NaOH (0.1 M). The resulting solution was quickly evaporated under reduced pressure, and 0.1 M triethanolamine hydrochloride buffer, pH 7.6 (2.5 mL), containing EDTA (20 mM), Na₃AsO₄ (100 μ L of a solution of 0.5 M), and NAD⁺ (100 μ L of a solution of 50 mM) was added to the residue. The absorbance at 340 nm was recorded before and 30 min after the addition of glyceraldehyde-3-phosphate dehydrogenase (10 μ L of a solution of 19 mg/mL). The yield of phosphoglycerate prepared from glyceraldehyde phosphate was typically about 80%. The phosphoglycerate and glycerol phosphate was separated as described above.

Determination of Specific Radioactivity. Phosphoglycerate was assayed enzymatically as described in Maister et al. (1976). Glycerol phosphate was assayed enzymatically as described in Fletcher et al. (1976). Assays were performed on duplicate samples (400 μ L each) from column fractions. Radioactivity determinations were made on duplicate samples (200 μ L each) from column fractions. The specific radioactivity of phosphoglycerate and glycerol phosphate was calculated as the weighted average of the specific radioactivities of individually assayed column fractions. The weighting factor for a particular fraction was the reciprocal of the variance of the specific radioactivity of that fraction.

The accuracy of each enzymatic assay was checked by comparison with a colorimetric assay of phosphate monoester content. A sample (100 μ L) from a column fraction was incubated for 30 min at 20 °C with alkaline phosphatase (10 μ L of a solution of 1.6 mg/mL), and the product inorganic phosphate was visualized by the method of Ames (1966).

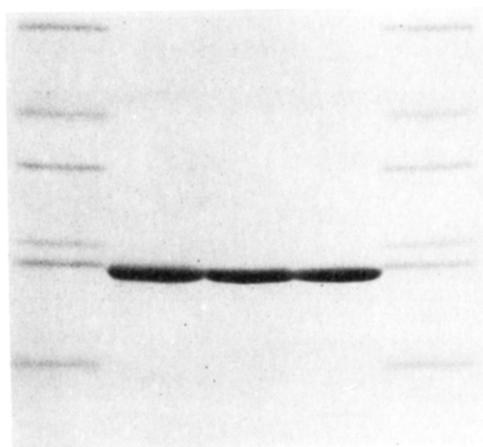
The nomenclature of Alberly and Knowles (1976a) is used to describe the results of the tritium experiments: the specific radioactivity of glycerol phosphate (or its progenitor dihydroxyacetone phosphate) is *s*, the specific radioactivity of phosphoglycerate (or its progenitor glyceraldehyde phosphate) is *p*, and the specific radioactivity of the solvent is *x*.

RESULTS

The purification of the mutant and wild-type isomerases from *E. coli* is summarized in Tables I and II, respectively. An analysis of the purified enzymes by polyacrylamide gel electrophoresis is shown in Figure 4. Protein sequence analysis of both the wild-type and mutant isomerases from *E. coli* indicated that about 20% of the subunits had a methionine

Table II: Purification of Wild-Type Triosephosphate Isomerase from *E. coli* Strain DF502(pX1)

step	$10^{-5} \times$ total units	sp act. (units/mg)	x-fold purification	yield (%)
crude lysate	5.35	40.2	1	100
ammonium sulfate supernatant	2.98	456	11	56
ammonium sulfate pellet	2.50	424	11	47
QAE-Sephadex	1.30	2970	74	24
Sephadex G-100	0.944	8910	222	18



mutant chicken TIM from coli
chicken TIM from coli
chicken TIM from chickens

FIGURE 4: Polyacrylamide gel electrophoresis of wild-type and mutant isomerases under denaturing conditions.

residue at the N-terminus. No methionine was found at the N-terminus of wild-type isomerase from chicken muscle, as expected (Furth et al., 1974).

The steady-state parameters for catalysis by the wild-type (Putman et al., 1972) and mutant (Straus et al., 1985) isomerases are presented in Table III. The value of k_{cat} was found to be essentially invariant between pH 6.6 and pH 8.6 for both the wild-type and the mutant isomerases with glyceraldehyde phosphate as substrate. The mutant enzyme was found to be equally as stereospecific as the wild-type isomerase. The amount of substrate available to the mutant enzyme in a solution of (RS)-glyceraldehyde phosphate was $98 \pm 3\%$ of that utilized by the wild-type isomerase. Wild-type enzyme removed $98.6 \pm 1.4\%$ of the tritium from a sample of dihydroxyacetone phosphate that had been labeled by wild-type isomerase and $98.6 \pm 5.6\%$ of the tritium from a sample of dihydroxyacetone phosphate that had been labeled by mutant isomerase. The solvent deuterium isotope effects on k_{cat} for the wild-type and mutant isomerases with glyceraldehyde phosphate as substrate were 1.5 ± 0.1 and 1.5 ± 0.2 , respectively. Each isomerase retained more than 90% activity after incubation for 72 h in deuterated buffer. Bromohydroxyacetone phosphate inactivates the mutant isomerase. The results of the titration of the wild-type and mutant isomerases by bromohydroxyacetone phosphate are shown in Figure 5. Phosphoglycolohydroxamate was a competitive inhibitor of both the wild-type and mutant isomerases with K_i values of $7.1 \pm 0.5 \mu\text{M}$ and $22 \pm \mu\text{M}$, respectively.

The extent of tritium transfer from [1(R)- ^3H]dihydroxyacetone 3-phosphate to the ultimate product phosphoglycerate, **p** (expressed as a fraction of the specific radioactivity of the substrate at the start of the reaction, s_0), by the mutant isomerase at 51% reaction is 0.021 ± 0.002 . The specific

Table III: Steady-State Parameters for Wild-Type and Mutant Triosephosphate Isomerase^a

constant ^b	wild-type	mutant	wild-type/ mutant
k_{cat}^S (s^{-1})	4.3×10^2	1.8 ± 0.1	240
K_m^S (mM)	0.97	1.8 ± 0.2	0.54
$K_m^S(\text{unhydr})$ (mM) ^c	0.57	1.1 ± 0.1	0.54
k_{cat}^P (s^{-1})	4.3×10^3	2.8 ± 0.2	1500
K_m^P (mM)	0.47	0.13 ± 0.01	3.6
$K_m^P(\text{unhydr})$ (mM) ^c	0.018	0.0051 ± 0.0003	3.6
K_{eq}^d	21	21 ± 3	1.0

^a Wild-type data are from Putman et al. (1972). Mutant data are from Straus et al. (1985). All experiments were performed in 0.1 M triethanolamine hydrochloride buffer, pH 7.6, at 30 °C. ^b S as superscript indicates that dihydroxyacetone phosphate was the substrate; P as superscript indicates that glyceraldehyde phosphate was the substrate. ^c Values for the unhydrated forms are calculated as described in Alberly and Knowles (1976b). ^d Equilibrium constants are calculated with the Haldane equation.

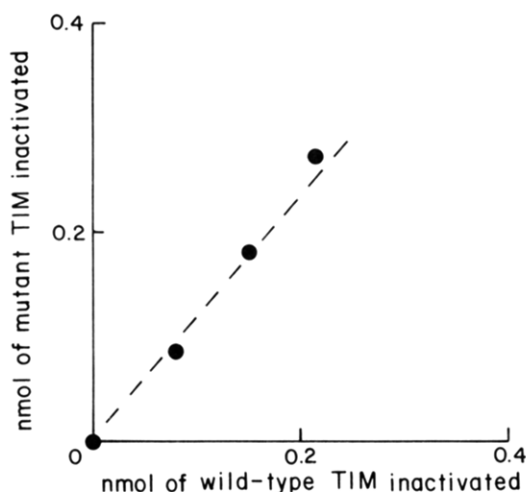


FIGURE 5: Plot of inactivation of wild-type (abscissa) and mutant (ordinate) isomerases by increasing amount of bromohydroxyacetone phosphate.

radioactivity of recovered substrate, **s** (expressed as a fraction of s_0), at 51% reaction is 1.4 ± 0.2 .

The location of the ^3H label in the product phospho[^3H]glycerate was established by washing out the hydrogen on C-2 into the solvent by treatment with phosphoglycerate mutase and enolase. When phospho[^3H]glycerate from the mutant isomerase catalyzed reaction of [1(R)- ^3H]dihydroxyacetone 3-phosphate was treated with phosphoglycerate mutase and enolase, more than 90% of the radioactive label washed into the solvent. When the same procedure was performed in the absence of phosphoglycerate mutase and enolase, more than 99% of the radioactive label remained in the nonvolatile residue. An upper limit of 10% can therefore be set on adventitious tritium on C-3 rather than on C-2 of the product phospho[^3H]glycerate.

The appearance of solvent tritium in substrate dihydroxyacetone phosphate and in product was determined at two extents of reaction catalyzed by the mutant isomerase. The values of the specific radioactivity of the remaining dihydroxyacetone phosphate, **s** (expressed as a fraction of the specific radioactivity of the solvent, **x**), as a function of the extent of the reaction, $1 - r$, are listed in Table IV and plotted in Figure 6. Discrimination against the appearance of solvent tritium in the ultimate product phosphoglycerate was determined at four extents of reaction. The values of the specific radioactivity of phosphoglycerate, **p** (expressed as a fraction of **x**), as a function of the extent of reaction are given in Table V.

Table IV: Dependence of Incorporation of Tritium into Remaining Dihydroxyacetone Phosphate on Extent of Mutant Isomerase Catalyzed Reaction in Tritiated Water^a

fractional extent of reaction ($1 - r$)	sp radioactivity of solvent (x) ($\mu\text{Ci}/\text{mmol}$)	sp radioactivity of remaining dihydroxyacetone phosphate (s) ($\mu\text{Ci}/\text{mmol}$)	tritium content of remaining dihydroxyacetone phosphate (s/x)
0.62	158 ± 1	16.1 ± 2.0	0.102 ± 0.013
0.86	121 ± 9	9.98 ± 0.05	0.082 ± 0.007

^a For details, see Experimental Procedures.Table V: Incorporation of Tritium into Product Phosphoglycerate during Mutant Isomerase Catalyzed Reaction of Dihydroxyacetone Phosphate in Tritiated Water^a

fractional extent of reaction ($1 - r$)	sp radioactivity of solvent (x) ($\mu\text{Ci}/\text{mmol}$)	sp radioactivity of phosphoglycerate (p) ($\mu\text{Ci}/\text{mmol}$)	isotopic content of product (p/x)	cor for incomplete exchange (c) ^b	A_8'
0.59	174 ± 15	96.2 ± 11.4	0.552 ± 0.080	0.001	0.553
0.62	158 ± 1	54.8 ± 5.9	0.347 ± 0.038	0.001	0.348
1.00	192 ± 10	97.2 ± 2.2	0.506 ± 0.029	0.000	0.506
1.00	210 ± 3	96.9 ± 1.8	0.462 ± 0.010	0.000	0.462
					0.467 ± 0.088^c

^a For details, see Experimental Procedures. ^b See eq AK6.9 in Appendix. ^c Mean value.Table VI: Dependence of Incorporation of Tritium into Remaining Glyceraldehyde Phosphate on Extent of Mutant Isomerase Catalyzed Reaction in Tritiated Water^a

fractional extent of reaction ($1 - r$)	sp radioactivity of solvent (x) ($\mu\text{Ci}/\text{mmol}$)	sp radioactivity of remaining glyceraldehyde phosphate (p) ($\mu\text{Ci}/\text{mmol}$)	tritium content of remaining glyceraldehyde phosphate (p/x)
0.15	159 ± 2	101 ± 1	0.63 ± 0.01
0.17	245 ± 16	144 ± 9	0.59 ± 0.05
0.31	190 ± 1	197 ± 4	1.04 ± 0.02
0.50	167 ± 3	161 ± 17	0.96 ± 0.10

^a For details, see Experimental Procedures.Table VII: Incorporation of Tritium into Product Phosphoglycerate during Mutant Isomerase Catalyzed Reaction of Glyceraldehyde Phosphate in Tritiated Water^a

fractional extent of reaction ($1 - r$)	sp radioactivity of solvent (x) ($\mu\text{Ci}/\text{mmol}$)	sp radioactivity of glycerol phosphate (s) ($\mu\text{Ci}/\text{mmol}$)	isotopic content of product (s/x)	cor for incomplete exchange (c) ^b	B_8'
0.17	245 ± 16	46.5 ± 1.0	0.190 ± 0.013	0.016	0.206
0.50	167 ± 3	17.7 ± 0.7	0.106 ± 0.005	0.005	0.111
					0.158 ± 0.067^c

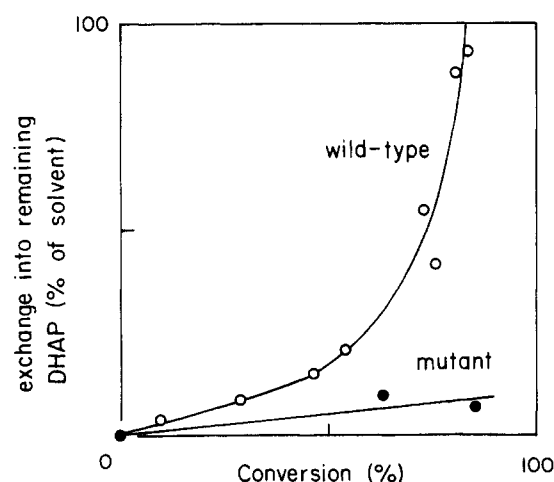
^a For details, see Experimental Procedures. ^b See eq AK6.9 in Appendix. ^c Mean value.

FIGURE 6: Exchange-conversion plot for wild-type (O) and mutant (●) isomerases with dihydroxyacetone phosphate as substrate. The data for the wild-type enzyme are from Maister et al. (1976).

The appearance of solvent tritium in substrate glyceraldehyde phosphate and in product was determined at four extents of reaction catalyzed by the mutant isomerase. The values of the specific radioactivity of the remaining glycer-

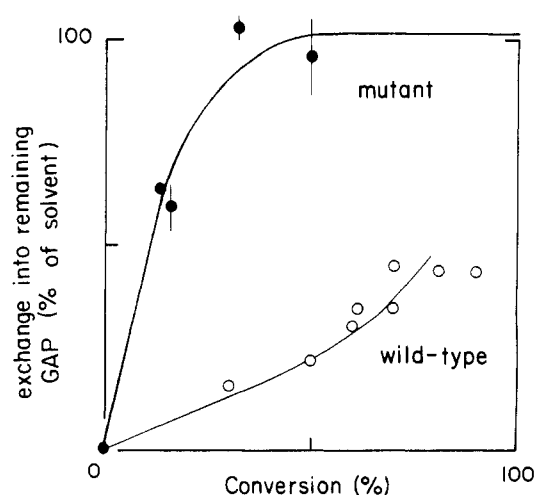


FIGURE 7: Exchange-conversion plot for wild-type (O) and mutant (●) isomerases with glyceraldehyde phosphate as substrate. The data for the wild-type enzyme are from Fletcher et al. (1976).

aldehyde phosphate, p (expressed as a fraction of x), as a function of the extent of the reaction, $1 - r$, are listed in Table VI and plotted in Figure 7. Discrimination against the appearance of solvent tritium in the ultimate product glycerol

phosphate was determined at two extents of reaction. The values of the specific radioactivity of glycerol phosphate, s (expressed as a fraction of x), as a function of the extent of reaction are given in Table VII.

The concentrations of phosphoglycerate and glycerol phosphate determined by enzymatic assay consistently agreed to within 10% with the concentrations determined by assay of the inorganic phosphate released by alkaline phosphatase.

DISCUSSION

To take a step toward defining the relationship between intermolecular distance and reaction rate for an enzyme-catalyzed reaction, we have used the methods of oligonucleotide-mediated site-directed mutagenesis to replace the glutamate residue at the active site of triosephosphate isomerase with aspartate. The gene for the enzyme from chicken breast muscle has been expressed from a cDNA clone in a strain of *E. coli* from which the endogenous bacterial isomerase has been deleted, and this gene was subjected to the mutagenic alteration. Three enzymes, wild-type isomerase from chicken muscle, wild-type isomerase expressed in *E. coli*, and mutant (Glu-165 to Asp) isomerase expressed in *E. coli*, have been purified to homogeneity and are indistinguishable on a denaturing polyacrylamide gel (Figure 4).

The necessary first step in the kinetic analysis of a mutant enzyme is to show that an observed change in catalytic activity is due solely to the targeted alteration. The catalytic activity of the mutant isomerase is about 10^3 -fold less than that of the wild-type enzyme (Table III). We established that the replacement of Glu-165 by Asp is responsible for the decrease in activity as follows. First, the whole of the mutated gene was sequenced, and the only differences found were those intentionally created by the synthetic oligonucleotide (Straus et al., 1985). Second, the N-terminus of the mutant protein was sequenced, and the mutant isomerase, like the wild-type enzyme expressed in *E. coli*, was found to contain an N-terminal methionine in about 20% of its subunits. From the crystal structure of the wild-type enzyme, the N-terminus of each subunit is on the surface of the protein, and the amino group of the N-terminal Ala residue is ~ 29 Å from C_β of Glu-165 (Banner et al., 1976). Since the wild-type enzyme from chicken muscle and that expressed in *E. coli* are kinetically indistinguishable, the occasional presence of an N-terminal methionine evidently does not affect the catalytic activity of the isomerase (Straus et al., 1985). Third, the possibility that the observed isomerase activity might result from contamination of a completely inactive mutant protein by a very small amount of wild-type isomerase was ruled out by the difference in the values of K_m for the wild-type and mutant enzymes (Table III). Since K_m (unlike V_{max}) is a property only of the type of active enzyme molecules, and not of the number of such molecules, we can be certain that the observed activity is due to mutant enzyme that has lower catalytic activity than the wild-type isomerase.

The next step in the analysis is to ensure that the change in catalytic activity is not a trivial consequence of pH, that is, that the mutation does not alter the pH dependence of the catalyzed reaction. In the present case, the amino acid change is conservative [the pK_a values for the side-chain carboxyl groups of Glu and Asp differ by only about 0.4 unit (Albert, 1952)], but pK_a values a protein can be grossly perturbed by the local environment (Schmidt & Westheimer, 1971). In fact, the pH variation of k_{cat} for the mutant and wild-type isomerases (Plaut & Knowles, 1972; Belasco et al., 1978) is identical between pH 6.6 and pH 8.6, indicating that the observed loss of activity does not result from changes in the

ionization behavior of catalytic groups.

We must also confirm that the mutant enzyme follows the same mechanistic pathway as the wild-type isomerase. The substitution of Asp for Glu-165 removes a methylene group from the isomerase active site, which may, for example, allow the substrates more freedom of motion when bound. Such increased flexibility in the active site could decrease the stereoselectivity of the reaction. The mutant isomerase might remove the 1-*pro-R* or the 1-*pro-S* proton from dihydroxyacetone 3-phosphate (the wild-type enzyme removes only the 1-*pro-R* proton), or it might isomerize (*R*)- or (*S*)-glyceraldehyde 3-phosphate [the wild-type enzyme isomerizes only (*R*)-glyceraldehyde 3-phosphate]. Each of these possibilities has been explored, and the mutant isomerase has been found to show the *same* stereochemical integrity as the wild-type enzyme.

The excision of a methylene group from the isomerase active site could have another mechanistic consequence. Affinity labeling studies (Waley et al., 1970; de la Mare et al., 1972) as well as crystal structures of the isomerase-substrate complex (Phillips et al., 1977; Alber et al., 1981) indicate that the carboxylate of Glu-165 abstracts a substrate proton directly from substrate. The replacement of Glu-165 with Asp creates a void in the active site that could in principle allow the carboxylate of Asp-165 to act as a general base through an intervening water molecule. This possibility was tested in two ways. First, we used solvent deuterium isotope effects to probe for an extra proton in flight in the transition state for substrate enolization in the mutant isomerase. Although the absolute magnitudes of solvent isotope effects are difficult to interpret because of unpredictable medium effects and possible structural changes in the enzyme, we can compare the values of $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ for the mutant and wild-type isomerases. The two enzymes show identical solvent deuterium isotope effects, which suggests that the mutant enzyme does not recruit a water molecule but—as the wild-type enzyme—abstracts a proton *directly* from the substrate. Second, if the mutant isomerase were to act through an intervening water molecule, then bromohydroxyacetone phosphate, a titrant for Glu-165 of the wild-type enzyme (de la Mare et al., 1972), would not inactivate the mutant isomerase as efficiently. Indeed, if Asp-165 acted as a general base, the mutant enzyme would also catalyze the *hydrolysis* of the bromo compound. The titration of the wild-type and mutant isomerases with bromohydroxyacetone phosphate is shown in Figure 5, which shows that bromohydroxyacetone phosphate inactivates the mutant and wild-type isomerases with the same efficiency.³ The mutant enzyme does not act on the bromo compound through an intervening water molecule.

We can now consider the reaction energetics of mutant triosephosphate isomerase. The derivation of an isomerase free-energy profile requires the combination of kinetic data from a number of different kinds of experiments that delineate the fate of tracer amounts of substrate tritium or solvent tritium and that allow the partitioning of the various forms of the liganded enzyme to be evaluated. The experiments are discussed here nonalgebraically, in order to provide a qualitative view of the way in which the kinetic information is obtained. The quantitative analysis leading to the free-energy profile for the reaction catalyzed by the mutant isomerase is given in the Appendix.

The extent to which a tritium label is transferred from the substrate to the product (a "transfer" experiment) is a measure

³ This experiment also shows that the mutant enzyme is a homogeneous preparation of enzyme species of low activity.

of the efficiency of the isotope exchange with solvent at the stage of the enediol intermediate. When $[1(R)\text{-}^3\text{H}]$ dihydroxyacetone 3-phosphate is used as the substrate for mutant isomerase in unlabeled solvent, 98% of the substrate tritium is lost to solvent. Thus, the conjugate acid of the enzymic base that abstracts the proton from substrate rapidly exchanges this proton with the solvent. This result is not surprising considering that the much more active wild-type enzyme also has time to equilibrate the abstracted proton with solvent almost completely, at the enediol stage (Herlihy et al., 1976).

The accumulation of tritium in the remaining substrate during a transfer experiment using tritiated substrate is a measure of the extent to which the proton-transfer step is rate-limiting in the reaction *up to* the enediol intermediate. When $[1(R)\text{-}^3\text{H}]$ dihydroxyacetone 3-phosphate is the substrate for mutant isomerase, tritium accumulates in the starting material as a result of the preferential consumption of unlabeled substrate by the mutant enzyme. The abstraction of the 1-*pro-R* proton must therefore limit the rate of the mutant isomerase catalyzed conversion of dihydroxyacetone phosphate to the enediol intermediate.

The discrimination against the appearance of solvent tritium in the product (a "discrimination" experiment) measures the extent to which the proton-transfer step is rate-limiting in the steps *after* the enediol intermediate. For example, when glyceraldehyde phosphate is the substrate for the wild-type enzyme in tritiated water, the specific radioactivity of the product is about 11% that of the solvent (Fletcher et al., 1976). This 9-fold discrimination against tritium suggests that the protonation of the enediol intermediate to form enzyme-bound dihydroxyacetone phosphate is rate-limiting in the conversion of the enediol intermediate to product. When this experiment is performed with the mutant isomerase, the specific radioactivity of the product is about 16% that of the solvent (Table VII). As in the case of the wild-type enzyme, this large discrimination against tritium indicates that the protonation of the enediol to give bound dihydroxyacetone phosphate is essentially irreversible. In the reverse direction (when unlabeled dihydroxyacetone phosphate is the substrate in tritiated water), the specific radioactivity of the product is about 80% that of the solvent when wild-type enzyme is used (Maister et al., 1976). This 1.3-fold discrimination against tritium was interpreted in terms of the almost complete equilibration in the protonation of the enediol intermediate at C-2 (that is, the bound enediol and the bound glyceraldehyde phosphate are essentially at equilibrium), followed by a slower step not involving proton transfer (Figure 3). When the analogous experiment is performed with mutant isomerase, the specific radioactivity of the product phosphoglycerate is about 47% that of the solvent: there is a 2-fold discrimination against tritium (Table V). While this effect is small, we believe that it does represent the intrinsic kinetic isotope effect. Although a kinetic isotope effect smaller than the intrinsic will be observed if a step other than proton transfer is rate-limiting, this explanation is not persuasive in the present case because the only isotopically insensitive step that could modulate the isotope effect on the protonation of the enediol to glyceraldehyde phosphate is the product "off" step. Yet for this transition state to become partially rate limiting for the mutant isomerase, glyceraldehyde phosphate would have to bind some 10^2 -fold more slowly to the mutant enzyme than to wild-type isomerase. This change is unreasonably large considering the conservative nature of the mutation.

There are several reasons why the intrinsic kinetic isotope effect for the enolization of glyceraldehyde phosphate could

be small. For instance, if the transition state for proton transfer is asymmetric, the isotope effect will be small because the zero-point energy of the starting material or of the product is maintained in the transition state. A small intrinsic isotope effect can also indicate a nonlinear transition state in which a bending mode rather than a bond stretching is converted into translational motion. A nonlinear transition state gives a small isotope effect because the vibrational frequencies (and hence the zero-point energies) for bending are much lower than those for stretching (Westheimer, 1961). Intrinsic tritium isotope effects for such processes are likely to be on the order of 3–5 (Hawthorne & Lewis, 1958; Winstein & Takahashi, 1958; Collins et al., 1959). Although we do not yet have a crystal structure of the mutant isomerase complexed with substrate, the structure of the wild-type enzyme–substrate complex shown in Figure 2 suggests that when Glu-165 is replaced by Asp, the carboxylate is likely to be relocated in a direction that is orthogonal to the C–H bond on C-2 of glyceraldehyde phosphate (the carbon that receives a proton to form glyceraldehyde phosphate). The view that the observed kinetic isotope effect is intrinsic is consistent with the available structural data on the enzyme.⁴

On the basis simply of the isotope transfer and isotope discrimination experiments discussed above, we have a qualitative picture of the relative heights of several of the transition states in the mutant isomerase catalyzed reaction. Thus, we know that transition state 1 is lower than transition state 2 and that transition state 4 is lower than transition state 3 (Figure 3). This qualitative analysis would nearly be complete if we knew the relative heights of transition states 2 and 3, that is, how the enediol intermediate partitions between starting material and product. To determine the relative heights of transition states 2 and 3, we again exploit the possibility of feeding solvent tritium into the middle of the isomerase reaction pathway, at the stage of the enediol intermediate.

The appearance of solvent tritium in remaining starting material (an "exchange-conversion" experiment) is a measure of the partitioning of the enediol intermediate between the forward reaction to product and the back reaction to substrate. In an exchange-conversion experiment, the isomerase reaction is run under irreversible conditions with initially unlabeled substrates in tritiated water, and the reaction is then quenched at a known extent of conversion of substrate to product. For example, the reaction of unlabeled dihydroxyacetone phosphate is run in tritiated water to determine how the enediol intermediate partitions between "exchange" back to substrate (to give *tritiated* dihydroxyacetone phosphate) and "conversion" to product (glyceraldehyde phosphate, which is swept on by the coupling enzyme to phosphoglycerate). For the wild-type enzyme, this experiment produced the exchange-conversion graph shown in Figure 6, which was interpreted as follows (Maister et al., 1976). At the start of the reaction, the slope of the line is about 0.3, indicating that the enediol intermediate undergoes conversion to product about 3 times for every time that it suffers exchange, having picked up tritium to return to dihydroxyacetone phosphate. At later times, the specific radioactivity of the remaining starting material rises increasingly, because the kinetic isotope effect in the processing of dihydroxyacetone phosphate leads to the preferential consumption of ^1H substrate, leaving the ^3H substrate behind.

⁴ A referee has suggested that the mutant enzyme might undergo a relatively slow conformational change that brings the carboxylate base close to the substrate, followed by a relatively fast proton transfer. The present results cannot eliminate such a possibility.

When this exchange-conversion experiment is performed with the mutant enzyme, essentially *no* radioactivity is found in remaining dihydroxyacetone phosphate (see Figure 6). The enediol intermediate now partitions forward (crossing transition states 3 and 4) more than 30 times for every time that it returns to dihydroxyacetone phosphate. The results of this exchange-conversion experiment lead to an unambiguous conclusion: the higher of transition states 3 and 4 is lower than the higher of transition states 1 and 2 in the free-energy profile of the mutant isomerase catalyzed reaction (Figure 3).

When the exchange-conversion experiment is performed in the opposite direction, with glyceraldehyde phosphate as substrate, the results shown in Figure 7 are obtained. For the wild-type enzyme, the initial slope of the line is about 0.3, indicating that the intermediate partitions forward to product dihydroxyacetone phosphate about 3 times for every time that it returns (having picked up tritium) back to glyceraldehyde phosphate. With the mutant isomerase, the exchange-conversion plot with glyceraldehyde phosphate as substrate indicates that the enediol intermediate partitions back to substrate (to give tritiated glyceraldehyde phosphate) about 6 times for every time it goes on to product (see Figure 7). This result tells us how much higher transition state 2 is than transition state 3.

The above discussion provides a qualitative understanding of the reaction energetics of the mutant enzyme. In the Appendix, the results of the transfer, discrimination, and exchange-conversion experiments, together with the steady-state kinetic parameters, are used to calculate the individual rate constants for the mutant isomerase catalyzed reaction. The algebraic strategy can be summarized as follows. First, the relative free energies of the two Michaelis complexes are calculated from the K_m values of the substrate and product. [This simplification could not, of course, be used in the analysis of the energetics of the wild-type enzyme (Albery & Knowles, 1976b).] Second, as with wild-type isomerase, the results of the tritium experiments are used to calculate the relative free energies of the transition states in the reaction. Finally, the free energy of the enediol intermediate is approximated by comparing the binding constant of the competitive inhibitor phosphoglycolohydroxamate to the wild-type and mutant enzymes. This inhibitor is believed to be a structural mimic of the enediol intermediate, which has been shown by X-ray crystallography to bind to the wild-type enzyme in a position indistinguishable from that of substrate (Davenport, 1986). The data thus obtained are illustrated as a free-energy profile in Figure 3.

Wild-type triosephosphate isomerase has reached the end of its evolutionary development as a catalyst (Albery & Knowles, 1976c, 1977; Knowles & Albery, 1977). The enzyme is "perfect" in that, at the ambient concentration of substrates *in vivo*, further evolutionary change could not result in any catalytic improvement. Our mutation has made the enzyme less perfect, taking, in a sense, an evolutionary step backward. The two enolization steps in the reaction have each been slowed by more than 3 orders of magnitude, and the overall reaction is now limited by a chemical transition state that involves covalency changes rather than one that involves substrate diffusion. Interestingly, the free energies of the bound intermediates (that is, the complexes of enzyme with substrate, with enediol, and with product) have changed little compared to the wild-type enzyme. The free energies of the transition states, however, have been seriously affected. Several molecular interpretations of this decrease in transition state binding can be proposed. From the viewpoint of Pauling

(1946, 1948), the "binding" of the transition states for enolization has decreased by about 4 kcal/mol. Protein fluctuations that occasionally push the enzymic base and the substrate together would then play an important role in catalysis by the mutant enzyme (Karplus & McCammon, 1983). Another interpretation involves the geometry of proton abstraction by the enzymic base. As a result of the preference for a proton to bond to *both* oxygen atoms in a carboxylic acid, Gandour (1981) has postulated that a carboxylate group is about 100-fold better as a catalyst when the proton is delivered to both carboxylate oxygens simultaneously ("syn") than when the proton is delivered to only one carboxylate oxygen ("anti"). As shown in Figure 2, the carboxylate of Glu-165 appears to abstract a substrate proton in a syn orientation in the wild-type enzyme. If Asp-165 of the mutant isomerase were to abstract a substrate proton from an anti orientation, a decrease in catalytic activity would be expected, even in the absence of other geometrical effects. Any more definitive statement must await a high-resolution crystal structure of the mutant isomerase-substrate complex. We are hopeful, however, that we shall eventually be able to correlate structural changes in the mutant isomerase with the kinetic behavior described above.

Though the replacement of Glu-165 with Asp has almost certainly not recreated an actual intermediate in the evolutionary development of triosephosphate isomerase, the mutant enzyme does provide a basis from which to investigate the forward evolution of an enzyme. By applying selective pressure to cells harboring the mutant enzyme, we shall now search for double mutants of increased catalytic activity (Dalbadie-McFarland et al., 1986).

APPENDIX

The discussion above has presented a qualitative picture of the phenomena that were observed. Quantitative information about the energetics of the reaction catalyzed by the mutant isomerase can be obtained from the treatment of Albery & Knowles (1976a). In this analysis, each type of isotopic experiment is given a three-letter description: the first letter specifies the isotope; the second letter defines the reactant (with a prime denoting that the reactant is isotopically labeled); the third letter describes the type of experiment [v is an initial velocity measurement; s or p indicates that the isotopic composition of the substrate S (here, dihydroxyacetone phosphate) or the product P (here, glyceraldehyde phosphate) is measured as a function of the extent of reaction $(1 - r)$]. The experimental results yield values for parameters called A_n (for experiments run from S to P) and B_n (for experiments run from P to S). The A_n and B_n parameters are functions of the individual rate constants and fractionation factors, and these parameters can be manipulated to extract the fractionation factors and individual rate constants (Albery & Knowles, 1976b).

The free-energy profile for the reaction catalyzed by wild-type enzyme is overdetermined in that several of the parameters are derived from more than one experiment. Although the analysis of mutant isomerase given here does not provide the extensive cross-checks that were possible in the analysis of wild-type enzyme (Albery & Knowles, 1976b), the results are sufficient to define the free-energy profile for the reaction catalyzed by the mutant isomerase. Of the 16 different experiments listed in Table II of Albery and Knowles (1976a), eight have been performed with mutant isomerase: HSv , HPv , $TS's$, $TS'p$, TSs , $TS'p$, TPs , and TPp . These eight experiments yield the values of A_1 , A_2 , A_5' , A_6' , A_7' , and A_8' and the corresponding B_n parameters. The equations used in the analysis are derived in Albery & Knowles (1976a), and the

labels assigned here (for example, AK0.00) refer to the equation numbers in that paper.

The fate of tritium in specifically labeled [1(*R*)-³H]dihydroxyacetone 3-phosphate during the course of a mutant isomerase catalyzed isomerization of this substrate was determined. There are three possible locations for the tritium label after partial reaction: in unreacted substrate, in the product, or in the solvent. The specific radioactivity of the substrate after partial reaction was determined (a TS's experiment). The equation for this type of experiment is

$$\ln(s/s_0) = (A_6' - 1) \ln r \quad (\text{AK5.11})$$

where *s* is the specific radioactivity of the dihydroxyacetone phosphate when a fraction *r* of it remains (at *r* = 1, *s* = *s*₀). With mutant enzyme, when *r* = 0.49, *s*/*s*₀ = 1.4 ± 0.2, giving a value for *A*₆' of 0.53 ± 0.22. Another site for the tritium label is in the ultimate product phosphoglycerate, and the specific radioactivity of phosphoglycerate after partial reaction was determined (a TS'p experiment). The equation for this type of experiment is

$$p/s_0 = A_5'(1 - r^{A_6'})/(1 - r) \quad (\text{AK5.12})$$

where the specific radioactivity of the product, *p*, is expressed as a fraction of the original specific radioactivity of the starting material, *s*₀. The parameter *A*₅' describes the extent to which label originally in *S* is retained in *P*. With mutant isomerase, when *r* = 0.49, *p*/*s*₀ = 0.021 ± 0.002, yielding a value for *A*₅' of 0.034 ± 0.006.

The appearance of solvent tritium in substrate dihydroxyacetone phosphate (a TSs experiment) and in product (a TS'p experiment) was determined. The equation for a TSs experiment is

$$s/x = A_7'(1 - r^{A_6'-1})/(A_6' - 1) \quad (\text{AK6.6})$$

where *s* is the specific radioactivity of the starting material, *x* is the specific radioactivity of the solvent, and *r* is the fraction of remaining reactant. The parameter *A*₇' is the initial gradient of the exchange-conversion profile shown in Figure 6; the parameter *A*₆' refers to the shape of the curve. Though in theory Figure 6 contains enough information to obtain both *A*₆' and *A*₇', the shape of the curve is not well-defined by the data, and we use the value of *A*₆' = 0.53 ± 0.22 from the TS's experiment. With a nonlinear least-squares computer program (Cleland, 1979) to fit the data in Table IV to eq AK6.6, the value of *A*₇' is 0.032 ± 0.013.

The equation for a TS'p experiment is

$$p/x = A_8' - c \quad (\text{AK6.9})$$

where

$$c = [A_5'A_7'/(A_6' - 1)][(r - r^{A_6'})/(1 - r)]$$

and *p* is the specific radioactivity of the ultimate product, phosphoglycerate. *c* is a small term describing the effect of incomplete isotopic equilibration of the enediol intermediate with the solvent. The parameter *A*₈' describes the fractionation of the tritium label that occurs in the steps after the enediol intermediate. From the data in Table V, the mean value of *A*₈' is 0.47 ± 0.09.

The parameters *B*₆' and *B*₇' were determined from the appearance of solvent tritium in substrate glyceraldehyde phosphate (a TPp experiment). The equation for a TPp experiment is

$$p/x = B_7'(1 - r^{B_6'-1})/(B_6' - 1) \quad (\text{AK6.6})$$

A nonlinear least-squares computer program (Cleland, 1979)

Table VIII: Values of Parameters from ¹H and ³H Experiments with Mutant Isomerase

From Straus et al. (1985)	
<i>A</i> ₁ = (6.1 ± 0.7) × 10 ⁻⁴ M s	<i>B</i> ₁ = (1.8 ± 0.2) × 10 ⁻⁶ M s
<i>A</i> ₂ = 0.56 ± 0.03 s	<i>B</i> ₂ = 0.36 ± 0.03 s
From Fletcher et al. (1976)	
<i>Φ</i> _S = 1.03 ± 0.04	<i>Φ</i> _P = 1.0 ± 0.2
From This Work	
<i>A</i> ₅ ' = 0.034 ± 0.006	<i>B</i> ₅ ' = 0.0048 ± 0.0025
<i>A</i> ₆ ' = 0.53 ± 0.22	<i>B</i> ₆ ' = 6.6 ± 1.5
<i>A</i> ₇ ' = 0.032 ± 0.013	<i>B</i> ₇ ' = 5.8 ± 1.2
<i>A</i> ₈ ' = 0.47 ± 0.09	<i>B</i> ₈ ' = 0.16 ± 0.07

Table IX: Values of *Φ*_{1,2}, *Φ*_{3,4}, and *θ* for Mutant Isomerase

<i>Φ</i> _{1,2} = 0.16 ± 0.07	<i>θ</i> = 0.062 (from <i>A</i> _n ' data)
<i>Φ</i> _{3,4} = 0.50 ± 0.10	<i>θ</i> = 0.13 (from <i>B</i> _n ' data)
	0.096 ± 0.048 (mean value)

was used to fit the data in Table VI to eq AK6.6 and yielded values of 6.6 ± 1.5 and 5.8 ± 1.2 for *B*₆' and *B*₇', respectively. With the values for the two substrate fractionation factors, *Φ*_S = 1.03 ± 0.04 and *Φ*_P = 1.0 ± 0.2 (Fletcher et al., 1976), the parameter *B*₅' can be calculated from

$$B_5' = (A_5'A_6'/B_6')(\Phi_S/\Phi_P) \quad (\text{AK7.1})$$

The value of *B*₅' determined in this way is 0.0048 ± 0.0025. Finally, the parameter *B*₈' was determined from the appearance of solvent tritium in the ultimate product, glycerol phosphate (a TPp experiment). The equation for a TPp experiment is

$$s/x = B_8' - c \quad (\text{AK6.9})$$

where

$$c = [B_5'B_7'/(B_6' - 1)][(r - r^{B_6'})/(1 - r)]$$

From the data in Table VIII, the mean value of *B*₈' is 0.16 ± 0.07. The values for all of the *A*_n and *B*_n are listed in Table IX.

The fractionation factors and individual rate constants for the reaction catalyzed by mutant triosephosphate isomerase are now obtained as follows. The fractionation factors *Φ*_{1,2} and *Φ*_{3,4} can be calculated from the values of *B*₈' and *A*₈', correcting for equilibration at the enediol intermediate with *B*₅' and *A*₅':

$$\Phi_{1,2} = B_8'/(1 - B_5') \quad (\text{AK6.10})$$

$$\Phi_{3,4} = A_8'/(1 - A_5') \quad (\text{AK6.10})$$

From the data in Table IX, the values of *Φ*_{1,2} and *Φ*_{3,4} are 0.16 ± 0.07 and 0.50 ± 0.10, respectively.

The parameter *θ* describes how the enediol intermediate partitions between *S* and *P* in the all-hydrogen system and can be calculated from either the *A*_n' or *B*_n' data with

$$\theta = \frac{A_7'}{\Phi_S A_6'(1 - A_5') - A_7'} = \frac{\Phi_P B_6'(1 - B_5') - B_7'}{B_7'} \quad (\text{AK7.5})$$

The values of *θ* from the *A*_n' and *B*_n' data are 0.062 and 0.13, respectively. The mean value of *θ* is 0.096 ± 0.048, which means that in the all-hydrogen system the enediol intermediate forms *P*, glyceraldehyde phosphate, about 10 times as frequently as it is converted to *S*, dihydroxyacetone phosphate.

The values of the fractionation factors, *Φ*_{1,2} and *Φ*_{3,4}, together with the value of the partition ratio, *θ*, suggest that the transition states flanking the enediol are the highest in the free-energy profile. Each Michaelis constant is therefore equal to a binding constant: *K*_m^S = *k*₋₁/*k*₁ and *K*_m^P = *k*₄/*k*₋₄. On the

Table X: Data for Free-Energy Profile of Mutant Triosephosphate Isomerase^a

value	ΔG (kcal/mol)
$k_1^b = 10^7 \text{ M}^{-1} \text{ s}^{-1}$	14.1 ^c
$k_{-1} = 1.1 \times 10^4 \text{ s}^{-1}$	12.2
$k_2 = 2.0 \text{ s}^{-1}$	17.3
$k_{-2} = 12 \text{ s}^{-1}$	16.3
$k_3 = 1.3 \times 10^2 \text{ s}^{-1}$	14.8
$k_{-3} = 33 \text{ s}^{-1}$	15.6
$k_4 = 1.9 \times 10^3 \text{ s}^{-1}$	13.2
$k_{-4}^b = 3.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$	12.0 ^c
$k_{-1}k_2k_3k_{-4}/(k_1k_2k_3k_4) = 330$	3.5

^a Determined at 30 °C. ^b From Alber and Knowles (1976b); values relate to the unhydrated substrate forms. ^c Calculated for a standard state of 40 μM .

basis that changing Glu-165 to Asp will have a negligible effect on the rate constants for the association of the enzyme with substrate and with product, then, as with wild-type isomerase, $k_1 \geq 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-4} = 3.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. By use of the data listed in Table III, the value of k_{-1} ($=k_1K_m^S$) is at least $1.1 \times 10^4 \text{ s}^{-1}$, and the value of k_4 ($=k_{-4}K_m^P$) is $1.9 \times 10^3 \text{ s}^{-1}$.

The partition ratios of the intermediates ($\alpha = k_2/k_{-1}$, $\beta = k_{-2}/k_3$, and $\gamma = k_{-3}/k_4$) can be derived from the A_n and B_n parameters, and these results can be used to calculate the remaining rate constants. Equation AK3.2 and the values of A_1 , k_1 , and θ can be used to calculate α ($=k_2/k_{-1}$):

$$A_1 = \frac{(1 + \alpha)(1 + \theta)}{\alpha k_1} \quad (\text{AK3.2})$$

The value of α is 1.8×10^{-4} , which yields a value for k_2 ($=k_{-1}\alpha$) of 2.0 s^{-1} . Similarly, eq AK3.2 and the values of B_1 and k_{-4} can be used to calculate γ ($=k_{-3}/k_4$):

$$B_1 = \frac{(1 + \gamma)(1 + \theta)}{\gamma \theta k_{-4}} \quad (\text{AK3.2})$$

The value of γ is 1.74×10^{-2} , which yields a value for k_{-3} ($=k_4\gamma$) of 33 s^{-1} . We now use eq AK1.7 to calculate β ($=k_{-2}/k_3$):

$$\theta = \frac{\beta(1 + \gamma)}{1 + \alpha} \quad (\text{AK1.7})$$

The value of β is 0.0944. In order to obtain the individual rate constants that comprise β ($=k_{-2}/k_3$), we require an estimate of the relative free energy of the enediol intermediate. The binding of phosphoglycolohydroxamate is presumed to mimic that of the enediol intermediate, and the K_i values for phosphoglycolohydroxamate with the wild-type and mutant isomerases are $7.1 \pm 0.5 \mu\text{M}$ and $22 \pm 1 \mu\text{M}$, respectively. In the absence of a better model, we assume that the enediol intermediate, like phosphoglycolohydroxamate, binds to the wild-type enzyme about 3 times more tightly than it binds to the mutant isomerase. From the rate constants in Knowles and Alber (1977), the equilibrium constant $k_1k_2/k_{-1}k_{-2}$ for the wild-type enzyme is 480 M^{-1} , implying that the analogous equilibrium constant is about 150 M^{-1} for the mutant isomerase. Using the value of this equilibrium constant and the previously determined values of k_1 , k_{-1} , and k_2 , we find that k_{-2} is 12 s^{-1} , and that k_3 ($=k_{-2}/\beta$) is $1.3 \times 10^2 \text{ s}^{-1}$.

The individual rate constants for the reaction catalyzed by mutant triosephosphate isomerase are listed in Table X. The free-energy changes corresponding to these rate constants at 30 °C were calculated for a standard state concentration of 40 μM , the concentration of triosephosphates in vivo (Williamson, 1965). These free-energy changes are listed in Table X and illustrated as a free-energy profile in Figure 3.

Finally, we can provide a cross-check on the value of the fractionation factor $\Phi_{3,4}$, which was found from tritium discrimination experiments to be 0.50 ± 0.10 . Both the initial gradient (described by B_6') and the curvature (described by B_7') of the exchange-conversion profile shown in Figure 7 depend on the ability of the enediol intermediate to return back to substrate glyceraldehyde phosphate having picked up tritium. Taken together, these parameters can be used to calculate θ , the partitioning for the all-hydrogen system (eq AK 7.5). Taken separately, however, each of these parameters depends on the fractionation factor $\Phi_{3,4}$ according to

$$B_6' = \frac{\Phi_{3,4}(1 + \theta^{-1})}{\Phi_P(1 + B_5'/\theta')} \quad (\text{AK5.3})$$

$$B_7' = \frac{\Phi_{3,4}(1 - B_5')}{\theta(1 + B_5'/\theta')} \quad (\text{AK6.5})$$

where θ' describes how the enediol partitions in a tritiated system. From the data in Tables VIII and IX, the ratio B_5'/θ is equal to 0.050. Since the ratio θ/θ' cannot be more than the intrinsic tritium kinetic isotope effect on one of the enolization steps, we will assume that the ratio B_5'/θ' is negligible compared to unity. Then, inserting the values in Tables VIII and IX into eq AK5.3 and into eq AK6.5 gives lower limits on $\Phi_{3,4}$ of 0.58 and 0.56, respectively. The agreement between these values, and that of 0.50 derived from the tritium discrimination experiment, is gratifying.

Registry No. TIM, 9023-78-3; L-Glu, 56-86-0; L-Asp, 56-84-8; bromohydroxyacetone phosphate, 24472-75-1; phosphoglycolohydroxamate (dicyclohexylammonium salt), 104196-61-4.

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