Triosephosphate Isomerase: Removal of a Putatively Electrophilic Histidine Residue Results in a Subtle Change in Catalytic Mechanism†

Elliott B. Nickbarg,‡,§ Robert C. Davenport,‖,⊥ Gregory A. Petsko,‖ and Jeremy R. Knowles*,‡

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received January 12, 1988; Revised Manuscript Received March 21, 1988

ABSTRACT: An important active-site residue in the glycolytic enzyme triosephosphate isomerase is His-95, which appears to act as an electrophilic component in catalyzing the enolization of the substrates. With the techniques of site-directed mutagenesis, His-95 has been replaced by Gln in the isomerase from *Saccharomyces cerevisiae*. The mutant isomerase has been expressed in *Escherichia coli* strain DF502 and purified to homogeneity. The specific catalytic activity of the mutant enzyme is less than that of wild type by a factor of nearly 400. The mutant enzyme can be resolved from the wild-type isomerase on nondenaturing isoelectric focusing gels, and an isomerase activity stain shows that the observed catalytic activity indeed derives from the mutant protein. The inhibition constants for arsenate and for glycerol phosphate with the mutant enzyme are similar to those with the wild-type isomerase, but the substrate analogues 2-phosphoglycolate and phosphoglycolohydroxamate bind 8- and 35-fold, respectively, more weakly to the mutant isomerase. The mutant enzyme shows the same stereospecificity of proton transfer as the wild type. Tritium exchange experiments similar to those used to define the free energy profile for the wild-type yeast isomerase, together with a new method of analysis involving ^{14}C and ^{3}H doubly labeled substrates, have been used to investigate the energetics of the mutant enzyme catalyzed reaction. When the enzymatic reaction is conducted in tritiated solvent, the mutant isomerase does not catalyze any appreciable exchange between protons of the remaining substrate and those of the solvent *either* in the forward reaction direction (using dihydroxyacetone phosphate as substrate) *or* in the reverse direction (using glyceraldehyde phosphate as substrate). However, the specific radioactivity of the product glyceraldehyde phosphate formed in the forward reaction is 31% that of the solvent, while that of the product dihydroxyacetone phosphate formed in the reverse reaction is 24% that of the solvent. The deuterium kinetic isotope effects observed with the mutant isomerase using [1(*R*)-^{2}H]dihydroxyacetone phosphate and [2-^{2}H]glyceraldehyde 3-phosphate are 2.15 ± 0.04 and 2.4 ± 0.1, respectively. These results lead to the conclusion that substitution of Gln for His-95 so impairs the ability of the enzyme to stabilize the reaction intermediate that there is a change in the pathways of proton transfer mediated by the mutant enzyme. The data allow us more closely to define the role of His-95 in the reaction catalyzed by the wild-type enzyme, while forcing us to be alert to subtle changes in mechanistic pathways when mutant enzymes are generated.

Although the combination of results from kinetic and structural experiments on triosephosphate isomerase has suggested some of the reasons behind the high catalytic efficiency of this enzyme, a complete understanding of the enzymatic mechanism and of the contributions to catalysis is still not available. In terms of the enzymic functionalities directly involved in catalysis, both a basic group (Glu-165) and one or more electrophilic groups (His-95 and/or Lys-12) appear to mediate the catalyzed reaction. As far as the enzyme base is concerned, the stereospecificity of proton removal and the presence of a small amount of proton transfer from substrate [1(*R*)-^{3}H]dihydroxyacetone phosphate[1] to product glyceraldehyde 3-phosphate suggest that a single enzymic base mediates the proton transfer process between the carbon centers (Rose, 1962; Herlihy et al., 1976). Inactivation of the isomerase by the affinity reagents glycidol phosphate (Waley et al., 1970) and bromohydroxyacetone phosphate (de la Mare et al., 1972) singled out the carboxylate of Glu-165 as the likely active-site base. Later crystallographic studies of the chicken muscle isomerase at 2.5-Å resolution supported this assignment (Banner et al., 1975). Recent site-directed mutagenesis experiments with the chicken enzyme have produced a mutant in which an aspartic acid residue replaces the active-site Glu-165 (Straus et al., 1985). While this change presumably moves the carboxylate group by something less than 1 Å, the effects upon the energetics of the reaction are dramatic (Raines et al., 1986). The rates of the enzyme-catalyzed enolization steps are each slowed by approximately 1000-fold.

It always seemed likely, however, that catalysis by a single base would be inadequate to account for the remarkable catalytic efficiency of the native enzyme. Triosephosphate isomerase accelerates the interconversion of its substrates by a factor of about 10^{9}-fold compared to catalysis by acetate ion (Hall & Knowles, 1975; Richard, 1984), and by analogy with the bifunctional acid–base catalysis of the enolization of

† This work was supported by the National Institutes of Health.

‡ Harvard University.

§ Present address: Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104.

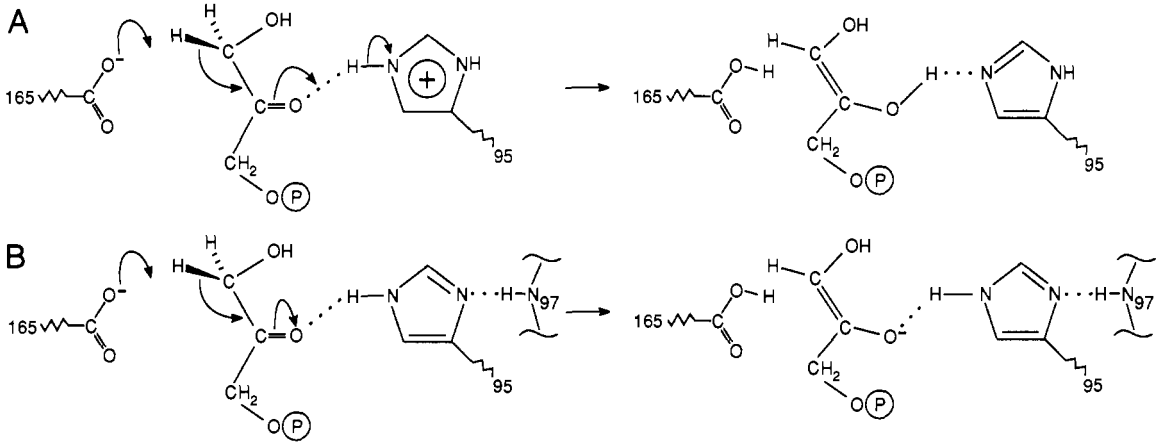
‖ Massachusetts Institute of Technology.

⊥ Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

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

0006-2960/88/0427-5948$01.50/0

Scheme I: (A) Catalysis in Wild-Type Triosephosphate Isomerase Using Glu-165 as a Base and His-95 as a General Acid Catalyst; (B) Catalysis in Wild-Type Triosephosphate Isomerase Using Glu-165 as a Base and His-95 as an Electrostatic Catalyst



acetone (Hegarty & Jencks, 1975), one might expect the enzyme to stabilize the developing negative charge on the substrate's carbonyl oxygen by the appropriate positioning of an electrophilic group in the active site. The existence of such an electrophilic component in the isomerase-catalyzed reaction is supported by experiments indicating that the enzyme polarizes substrate carbonyl groups. Thus the rate of reduction of the carbonyl group of dihydroxyacetone phosphate by borohydride is accelerated by about eightfold upon substrate binding to the enzyme (Webb & Knowles, 1974, 1975), despite a presumably lower accessibility of the carbonyl group when bound at the active site. Further, direct observation of enzyme-bound dihydroxyacetone phosphate by Fourier transform infrared spectroscopy revealed that the stretching frequency of the carbonyl group decreases by 19 cm^{-1} relative to that of the substrate free in solution (Belasco & Knowles, 1980), again suggesting that the isomerase polarizes the substrate carbonyl, presumably through interaction with an enzymic electrophile.

All of these results are consistent with crystallographic data obtained at 2.5-Å resolution from the triosephosphate isomerase from chicken muscle (Banner et al., 1975; Alber et al., 1981). Further, the structures of complexes of the yeast enzyme with substrate dihydroxyacetone phosphate (Alber et al., 1981) and with the competitive inhibitor phosphoglycolohydroxamate (R. Davenport, B. Seaton, G. Petsko, and D. Ringe, unpublished results) have been solved at 3.5- and 1.9-Å resolution, respectively. The models derived from these results place the carbonyl oxygens of the substrates within hydrogen-bonding distance of the side chains of His-95 and Lys-12 (this is equivalent to Lys-13 in the chicken isomerase), and electrophilic catalysis involving one or both of these residues seems likely. The spectroscopic work (Belasco & Knowles, 1980) and the kinetic studies (Webb & Knowles, 1974, 1975) that indicate an electrophilic component in the active site are consistent with catalysis by hydrogen bonding from an enzymic general acid that would yield an enzyme-bound enediol intermediate (Scheme IA). The crystallographic results, however, point to the existence of a hydrogen bond between the δ-nitrogen of His-95 and the N–H group of the main-chain amide of Glu-97. It was therefore suggested that the *un*protonated imidazole ring of His-95 could catalyze substrate enolization by electrostatic stabilization (Alber et al., 1981). In this formulation, no proton transfer to oxygen from His-95 would occur in the formation of an enediol*ate* intermediate (Scheme IB) (Petsko et al., 1984). Early proton NMR studies also suggested that His-95 does not titrate between pH 5.4 and pH 9.0 (Browne et al., 1976), though no formal assignment of the NMR resonance of the nontitrating histidine residue was made. It must be recognized, however, that neither crystallographic studies nor spectroscopic results showing carbonyl polarization can distinguish between catalysis by proton transfer and catalysis by electrostatic stabilization.

To resolve some of these questions and to probe the role of His-95 in the catalytic mechanism, site-directed mutagenesis has been used to produce a mutant yeast triosephosphate isomerase containing a glutamine residue in place of His-95 (Alber & Kawasaki, 1982; Petsko et al., 1984; Davenport, 1986). We report here the effects on the mechanism and on the reaction energetics of changing this putative electrophilic functionality. It is evident that this change not only has a substantial effect on the specific catalytic activity but also produces a subtle but significant change in the reaction mechanism. These results sound a general alarm and emphasize the need for the careful mechanistic evaluation of mutant enzymes produced in site-directed mutagenesis experiments.

Experimental Procedures

Materials

Enzymes, substrates, inhibitors, cofactors, and chromatographic materials were purchased or prepared as described in Nickbarg and Knowles (1988). [1(*R*)-^{2}H]Dihydroxyacetone phosphate and [2-^{2}H]glyceraldehyde phosphate were prepared as described in Nickbarg and Knowles (1988). [1(*R*)-^{3}H]-Dihydroxyacetone phosphate was synthesized by R. Raines according to the procedure of Herlihy et al. (1976). Phospho[1-^{14}C]glycerate was synthesized by W. Guilford (Guilford et al., 1987) according to a modification of the procedure of Addadi et al. (1983). Isoelectric focusing gels were run on prepoured analytical PAGplates (1804-102, pH 4.0–6.5) from LKB Inc. (Gaithersburg, MD). Phenazine methosulfate and nitro blue tetrazolium were both from Aldrich (Milwaukee, WI). All other chemicals and reagents were obtained as described in Nickbarg and Knowles (1988).

A synthetic pentadecanucleotide complementary to 14 bases of the sense strand of the yeast isomerase coding sequence and encoding a change in the His-95 CAC codon to the Gln CAA codon in the eighth position was purchased from New England Biolabs (Beverly, MA). The M13 sequencing primer, all DNA restriction enzymes, Klenow fragment, polynucleotide kinase, and T4 DNA ligase were also from New England Biolabs. [γ-^{32}P]ATP[2] (5000 Ci/mol) was obtained from New England

Nuclear (Boston, MA). Coliphage M13 strain phTPI is a derivative of M13 mp18 (Messing, 1983) containing the entire coding region of the yeast isomerase gene from plasmid pTPIC10 (Alber & Kawasaki, 1982; Davenport, 1986). Plasmid p12 is a *amp*^{r},*tpi*$^+$ derivative of pUC12 (Vieira & Messing, 1982) and pTPIC10 (Davenport, 1986; Casal et al., 1986), which expresses yeast triosephosphate isomerase in *Escherichia coli* JM101 (Messing, 1983). JM101 was provided by Dr. Regina Reilly. DF-502, a streptomycin-resistant strain of *E. coli* that lacks the endogenous *E. coli* triosephosphate isomerase (Straus & Gilbert, 1985), was kindly provided by D. Fraenkel and was used as the expression system for both the p12 and the p8 mutant plasmids (described below).

Methods

Samples (5–1000 μL) for radiochemical analysis were dissolved in scintillation cocktail (6–12 mL) and counted on a Beckman LS1801 automatic liquid scintillation counter. Scintillation counting for both ^{3}H and ^{14}C was done after calibration with Beckman counting standards and the double-label counting programs supplied with the instrument. Growth media and measurements of ultraviolet/visible absorbance, pH, conductivity, protein concentration, and enzyme activity were made as described in Nickbarg and Knowles (1988). Base-labile inorganic phosphate was determined colorimetrically according to the following procedure. Samples (100 μL) in acid-washed glass tubes were evaporated to dryness in an oven at 105 °C. The samples were cooled and then made basic by the addition of 1 M NaOH (200 μL). The amount of inorganic phosphate was then determined as described by Chen and Toribara (1956). HPLC analysis of mixtures of radiolabeled 3-phosphoglycerate and glycerol phosphate was done on a Pharmacia Mono Q HR 5/5 column fitted into a Waters HPLC system consisting of a Model 680 gradient controller, two Model 510 pumps, and a U6K injector. The separation involved an isocratic wash (2 min) with 10 mM triethylammonium formate buffer, pH 3.8, containing EDTA (1 mM) followed by a linear gradient (10–500 mM over 15 min) of triethylammonium formate buffer, pH 3.8, containing EDTA (1 mM), at a flow rate of 2 mL/min. Fractions (1 mL) were collected directly into scintillation vials and then counted. Protein sequences were determined by automated Edman degradation with an Applied Biosystems 470A sequencer as described in Raines et al. (1986).

Mutagenesis. Primer-directed mutagenesis of purified virion phTPI DNA was done by the two-primer method of Norris et al. (1983), using the mutagenic and sequencing primers described above. Competent *E. coli* JM101 cells (Maniatis et al., 1982) were transformed with the mutagenized DNA. Individual phage plaques resulting from the transformation were screened by dot blotting with the mutagenic primer made radioactive by treatment with polynucleotide kinase and [γ-^{32}P]ATP (Zoller & Smith, 1983). Phage RF DNA was purified from positive clones, and a *Hin*dII–*Bgl*II-cut 95 base pair fragment containing the mutagenized site was isolated and used to replace the corresponding fragment in plasmid p12. The modified plasmid was used to transform JM101, and purified DNA from a subclone was sequenced according to Maxam and Gilbert (1980). One subclone, designated p8, was used to transform DF502.

Isoelectric Focusing. Isoelectric focusing of mutant and yeast wild-type triosephosphate isomerase was performed on an LKB Multiphor apparatus thermostated at 4 °C. Protein samples (5–10 μg) were applied to a small applicator strips (0.5 cm × 1 cm) directly on the gel surface. The gel was focused for 2 h, the pads were removed, and focusing was continued for an additional 2.5–4 h. To increase resolution, the electrode strips were arranged to form a pH gradient along the long (24 cm) axis of the gel. The gels were run at constant power (1 W/cm of width) to a maximum of 2500 V. The pH gradient was determined with a 1 mg/mL solution each of carbonic anhydrase (ex bovine erythrocytes, pI = 5.23), phycocyanin (ex *Spirulina platensis*, pI = 4.65), and β-lactoglobulin B (ex bovine milk, pI = 6.18) as standards (Sigma, St. Louis, MO). Gels were stained for triosephosphate isomerase activity according to the method of Scopes (1968), except that the reagent was in 100 mM triethanolamine hydrochloride buffer, pH 7.5, and additionally contained EDTA (1 mM), β-mercaptoethanol (1 mM), and dihydroxyacetone phosphate (25 mM). After activity staining, the gel was fixed and then stained for protein with Coomassie blue.

Steady-State Kinetics. Mutant isomerase activity was measured in the same fashion as that of wild-type yeast isomerase (Nickbarg & Knowles, 1988), except that higher levels of mutant isomerase were used.

Purification of Mutant Yeast Triosephosphate Isomerase. *E. coli* DF502(p8) cell paste (21 g, wet wt) was obtained from 12 L of culture grown to an A_{550nm} of 2.5 in shaker flasks in rich (LB) medium initially containing histidine (40 μg/mL), uracil (40 μg/mL), glycerol (0.2% v/v), and ampicillin (150 μg/mL). After 4.5 h of growth, when the culture had reached an A_{550nm} of 0.8, one additional starting equivalent each of histidine, uracil, glycerol, and ampicillin was added to each flask. The cells were harvested after a further 3.5 h of growth by centrifugation (30000*g*) in a Sorvall continuous-flow rotor at 4 °C. All subsequent purification steps were performed at 4 °C. All glassware was either new or acid washed, to minimize the possibility of any contamination of triosephosphate isomerase from other sources. The cell cake was suspended in 85 mL of 10 mM Tris-HCl buffer, pH 7.5, containing EDTA (1 mM) and dithioerythritol (1 mM), and then lysed by two passes through a French press at 10000 psi. The lysate was centrifuged for 30 min at 30000*g* to remove cell debris. Finely ground solid ammonium sulfate (38.3 g) was slowly added with stirring to the supernatant (89 mL). After the solution was stirred overnight, the precipitate was collected by centrifugation at 30000*g* for 30 min. The pellet was resuspended in 30 mL of Tris-HCl buffer, pH 7.5, containing EDTA (1 mM) and dithioerythritol (1 mM) and then dialyzed exhaustively against the same buffer. The supernatant (97 mL) was brought to 90% saturation in ammonium sulfate by the slow addition of finely ground ammonium sulfate (18.4 g) with stirring. After being stirred for 18 h, the precipitate was collected by centrifugation at 30000*g* for 30 min. The pellet was resuspended in 10 mL of 10 mM Tris-HCl buffer, pH 7.5, containing EDTA (1 mM) and dithioerythritol (1 mM) and the solution then dialyzed against the same buffer. Approximately one-third of the total isomerase activity was in the 65–90% ammonium sulfate fraction, while the remainder precipitated in the 0–65% fraction. The dialyzed 65–90% ammonium sulfate fraction (14 mL) was loaded onto a column (10 × 16 cm^2) of QAE-Sephadex A-50 equilibrated with 10

[2] Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; DEAE, diethylaminoethyl; DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetate; GAP, glyceraldehyde 3-phosphate; HPLC, high-pressure liquid chromatography; NAD$^+$, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; PGH, phosphoglycolohydroxamate; QAE, [diethyl(2-hydroxypropyl)amino]ethyl; Tris, tris(hydroxymethyl)aminomethane.

mM Tris-HCl buffer, pH 7.5, containing EDTA (1 mM) and β-mercaptoethanol (1 mM). The column was eluted with a linear gradient of KCl (0–100 mM, 500 + 500 mL) in the same buffer. Fractions (5 mL) were collected and assayed for enzymatic activity and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Fractions containing the isomerase were pooled and concentrated by ultrafiltration through an Amicon PM-10 filter membrane. The 0–65% ammonium sulfate fraction (40 mL) was then brought to 50% saturation by the addition of ammonium sulfate (12.52 g), followed by stirring for 1 h. After centrifugation at 30000*g* for 20 min, the isomerase was then recovered by bringing the fraction to 80% saturation with the addition of solid ammonium sulfate (8.56 g). After being stirred for 12 h, the precipitate was collected by centrifugation at 30000*g* for 30 min. The pellet was resuspended in 10 mL of 10 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (1 mM) and dithioerythritol (1 mM) and then dialyzed against 2 L of the same buffer. The isomerase was then purified by chromatography on QAE-Sephadex A-50, as described for the wild-type enzyme (Nickbarg & Knowles, 1988).

3-Phospho[2-^{3}H]glycerate. Tritiated 3-phosphoglycerate labeled at the C-2 position was prepared from glyceraldehyde phosphate as follows. The reaction mixture contained (in 31 μL) 200 mM triethanolamine hydrochloride buffer (10 μL), pH 7.5, containing EDTA (10 mM), sodium arsenate (1 μmol), NAD^+ (0.5 mg), chicken triosephosphate isomerase (990 units), (*RS*)-glyceraldehyde phosphate (0.18 μmol), and tritiated water (18 mCi). The mixture was left at room temperature for 1 h. Glyceraldehyde-3-phosphate dehydrogenase (4 units) was then added to convert the equilibrated mixture of triosephosphates into 3-phosphoglycerate. After incubation at room temperature for 3 h, the reaction was quenched by acidification to pH 2.0 with HCl (1 N, 5 μL). The solvent was then removed by bulb-to-bulb distillation. The residue was dissolved in 10 mM HCl (100 μL), and the solvent was again removed by distillation. This sequence was repeated once more. The residue was then taken up in water (1 mL), and the solution was filtered through an Amicon Centricon-10 ultrafiltration apparatus to remove isomerase and coupling enzymes. The filtrate was analyzed by HPLC on a Pharmacia Mono Q column as described above. Analysis showed that >95% of the tritium radioactivity coeluted with authentic 3-phospho[1-^{14}C]glycerate.

[1-^{14}C]Glyceraldehyde Phosphate and [2-^{3}H,1-^{14}C]-Glyceraldehyde Phosphate. Labeled glyceraldehyde phosphate was prepared from labeled 3-phosphoglycerate with phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase according to a modification of the procedures used to synthesize [2-^{2}H]glyceraldehyde 3-phosphate (Leadlay et al., 1976). The reaction mixture contained (in 2.4 mL) 100 mM imidazolium chloride buffer, pH 7.0, magnesium acetate (50 μmol), potassium acetate (100 μmol), phosphoenolpyruvate (5 mg), NADH (3.6 mg), ADP (0.2 mg), isomerase-free glyceraldehyde-3-phosphate dehydrogenase (80 units), pyruvate kinase (500 units), phosphoglycerate kinase (500 units), 3-phospho[2-^{3}H]glycerate (0.14 μCi), and 3-phospho[1-^{14}C]glycerate (0.024 μCi). The mixture was left at room temperature for 30 min and then diluted into cold 12 mM triethylammonium formate buffer, pH 3.8 (100 mL). The product was then purified by chromatography on AG 1X8, as previously described for [2-^{2}H]glyceraldehyde phosphate (Nickbarg & Knowles, 1988). Analysis of the purified sample by HPLC showed that >99% of the radioactivity comigrated with glyceraldehyde phosphate. A small portion (5 μL) of the sample was converted to 3-phosphoglycerate by treatment with glyceraldehyde-3-phosphate dehydrogenase, NAD^+, and arsenate. HPLC analysis of this portion confirmed that the radioactivity comigrated with authentic 3-phosphoglycerate. The product [2-^{3}H,1-^{14}C]glyceraldehyde phosphate had a $^3H/^{14}C$ ratio of 6.1, which was identical with that expected from the proportions of [2-^{3}H]- and [1-^{14}C]phosphoglycerate used in the synthesis. Glyceraldehyde phosphate labeled solely with ^{14}C was prepared from 3-phospho[1-^{14}C]glycerate under similar conditions.

[1-^{14}C]Dihydroxyacetone Phosphate. [1-^{14}C]Dihydroxyacetone phosphate was synthesized from [1-^{14}C]glyceraldehyde phosphate. The reaction mixture contained (in 2.46 mL) unlabeled dihydroxyacetone phosphate (15.2 μmol) adjusted to neutrality with 1 N NaOH prior to use, 200 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (10 mM), [1-^{14}C]glyceraldehyde phosphate (0.52 μCi), and wild-type chicken isomerase (950 units). The mixture was left for 3 h at room temperature. The isomerase was then removed by passage through a column (0.2 cm^2 × 3 cm) of Dowex 50W (H^+ form). The column was washed with water (4 mL), and the combined filtrate and washings were adjusted to pH 4 with 1 N NaOH and concentrated by evaporation in vacuo. The specific radioactivity of the equilibrium mixture of triosephosphates [containing 9.5 mM dihydroxyacetone phosphate and 0.3 mM (*R*)-glyceraldehyde phosphate] was 0.036 μCi/μmol. This mixture was used without further purification.

[1(R)-^{3}H,1-^{14}C]Dihydroxyacetone Phosphate. Doubly labeled [1(*R*)-^{3}H,1-^{14}C]dihydroxyacetone phosphate was prepared by combining samples of [1-^{14}C]dihydroxyacetone phosphate (0.036 μCi/μmol, 9.5 mM) and of [1(*R*)-^{3}H]dihydroxyacetone phosphate (9.1 μCi/μmol) to give doubly labeled dihydroxyacetone phosphate with a $^3H/^{14}C$ ratio of 5.0 after HPLC purification.

Stereospecificity: *(A) Dihydroxyacetone Phosphate as Substrate.* The stereospecificity of the proton exchange reaction catalyzed by the mutant isomerase was evaluated by incubation of dihydroxyacetone phosphate with the isomerase in tritiated water, as described earlier (Raines et al., 1986). Samples of the equilibrium mixture of tritiated triose phosphates that had been labeled by the mutant isomerase were incubated either with chicken triosephosphate isomerase or with rabbit muscle aldolase. A control reaction mixture contained no enzyme.

(B) Glyceraldehyde Phosphate as Substrate. The concentration of (*R*)-glyceraldehyde phosphate in a solution of racemic (*RS*)-glyceraldehyde phosphate was compared by use of either the mutant or the wild-type yeast isomerase, as described by Raines et al. (1986).

Incorporation of Solvent Tritium into Substrate (R)-Glyceraldehyde 3-Phosphate in the Presence of Glyceraldehyde-3-phosphate Dehydrogenase. A reaction mixture containing (in 1.32 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.5, EDTA (20 mM), sodium arsenate (10 mM), NAD^+ (5 mg), glyceraldehyde-3-phosphate dehydrogenase (80 units), and tritiated water (22.5 mCi) was equilibrated at 30 °C, and reaction was initiated by the addition of (*RS*)-glyceraldehyde phosphate (2.75 μmol). After incubation at 30 °C for 90 min, the reaction was quenched by acidification to pH 2 with HCl (1 M, 160 μL). Tritiated water and glyceraldehyde-3-phosphate dehydrogenase were removed as described above, and DEAE-cellulose chromatography was used to separate 3-phosphoglycerate from (*S*)-glyceraldehyde phosphate as previously described. The

column fractions were assayed for 3-phosphoglycerate and for radioactivity. A colorimetric assay for base-labile inorganic phosphate was used to detect (*S*)-glyceraldehyde phosphate (see above).

Reaction of [1(R)-^{2}H]Dihydroxyacetone Phosphate and of [2-^{2}H]Glyceraldehyde Phosphate. Kinetic experiments using [1(*R*)-^{2}H]dihydroxyacetone phosphate and [2-^{2}H]glyceraldehyde phosphate were done as described by Nickbarg and Knowles (1988).

Appearance of Solvent Tritium in Remaining Substrate and in Product. Isomerase-catalyzed reactions were run in each direction as described in Nickbarg and Knowles (1988), except that the reaction was initiated by the addition of mutant isomerase.

Fate of the Tritium Label from [1(R)-^{3}H]Dihydroxyacetone Phosphate. An isomerase-catalyzed reaction was run as described in Nickbarg and Knowles (1988), except that the reaction was initiated by the addition of mutant isomerase.

Fate of the Tritium Label from [1(R)-^{3}H,1-^{14}C]Dihydroxyacetone Phosphate. The reaction mixture contained (in 1.482 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.5, EDTA (5 mM), isomerase-free glyceraldehyde-3-phosphate dehydrogenase (80 units), NAD^+ (5 mg), sodium arsenate (10 mM), and [1(*R*)-^{3}H,1-^{14}C]dihydroxyacetone phosphate (1 μmol, 0.036 μCi of ^{14}C, $^3H/^{14}C$ = 5.0). The solution was mixed and equilibrated at 30 °C. A reference sample (200 μL) was removed and quenched in 1 N HCl (25 μL) before storage at −70 °C for later analysis. The absorbance of the reaction mixture at 340 nm was determined. The reaction was then initiated by the addition of mutant isomerase (2.5 units). A portion of the reaction mixture was transferred to a short-path (2 mm) optical cuvette for monitoring the absorbance at 340 nm. At four time intervals, a sample (200 μL) was withdrawn and quenched into 1 N HCl (25 μL). At the completion of the reaction, the sample in the optical cuvette was quenched and stored. After quenching, solvent was removed from each sample by bulb-to-bulb distillation. The sample residues were taken up in water (0.3 mL), adjusted to pH 4 with 0.1 N NaOH, and filtered through Amicon Centricon-10 ultrafiltration cones at 5000*g* to remove isomerase and glyceraldehyde-3-phosphate dehydrogenase. The mixtures of radiolabeled 3-phosphoglycerate and dihydroxyacetone phosphate were then separated by HPLC and analyzed for ^{3}H and ^{14}C as described above.

Fate of the Tritium Label from [2-^{3}H,1-^{14}C]Glyceraldehyde 3-Phosphate. The isomerase-catalyzed reaction mixture contained (in 2.96 mL) 66 mM triethanolamine hydrochloride, pH 7.5, EDTA (3.3 mM), isomerase-free glycerophosphate dehydrogenase (10 units), NADH (3 mg), (*RS*)-glyceraldehyde phosphate (4 μmol), and [2-^{3}H,1-^{14}C]glyceraldehyde phosphate (0.036 μCi of ^{14}C, $^3H/^{14}C$ = 5.5). The reaction mixture was equilibrated at 30 °C, and the absorbance at 340 nm was determined. A reference sample (500 μL) was removed, quenched into 1 N HCl (30 μL), and stored at −70 °C for later analysis. The reaction was then initiated by the addition of mutant isomerase (0.6 unit). A sample was transferred into a short-path (2 mm) optical cuvette to monitor the extent of reaction. At three points during the course of the reaction, a sample (500 μL) was withdrawn, quenched in 1 N HCl (30 μL), and stored. The reaction in the optical cuvette was followed to completion and then quenched and stored. Solvent was removed from each sample by bulb-to-bulb distillation. The sample residues were dissolved into water (1 mL), and the enzymes were removed by ultrafiltration. The samples were neutralized with 1 N NaOH, and the remaining glyceraldehyde phosphate in each sample was then converted to 3-phosphoglycerate by addition of NAD^+ (2 mg), sodium arsenate (10 μmol), and glyceraldehyde-3-phosphate dehydrogenase (16 units). Glyceraldehyde-3-phosphate dehydrogenase was then removed from each sample by ultrafiltration. The mixtures of radiolabeled 3-phosphoglycerate and glycerophosphate were then separated by HPLC and analyzed for ^{3}H and ^{14}C as described above.

Appearance of Solvent Tritium in Substrate [1-^{14}C]Dihydroxyacetone 3-Phosphate and in Product. A mutant isomerase catalyzed reaction was run under conditions similar to those used by Maister et al. (1976). The reaction mixture contained (in 1.5 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.5, EDTA (5 mM), NAD^+ (3 mM), sodium arsenate (10 mM), glyceraldehyde-3-phosphate dehydrogenase (80 units), tritiated water (45 mCi), and 1 mM [1-^{14}C]dihydroxyacetone phosphate (0.036 Ci/mol). The initial absorbance at 340 nm was determined, and duplicate samples (5 μL) were removed and diluted into water (1.0 mL) for later determination of the solvent tritium specific radioactivity. A third sample (250 μL) was removed, brought to pH 2 with 25 μL of 1 N HCl, quickly frozen in liquid nitrogen, and stored at −70 °C. The reaction mixture was equilibrated at 30 °C, and the reaction was initiated by the addition of mutant yeast isomerase (2.5 units). A portion (500 μL) of the reaction mixture was transferred to a short-path (2 mm) optical cuvette in order to monitor the extent of reaction at 340 nm. The remainder of the reaction mixture was separately incubated at 30 °C. At three stages in the reaction, a sample (250 μL) was removed and quenched into 1 N HCl (25 μL). Each sample was frozen in liquid nitrogen and stored. Solvent was then removed from each sample by bulb-to-bulb distillation. The distillation residues were dissolved once in 0.8 mM HCl (0.5 mL), and the solvent was again removed by distillation. The residues were each then dissolved in water (0.5 mL), and the solvent was again removed. The samples were then dissolved into water (0.5 mL), and the enzymes were removed by ultrafiltration. The samples were adjusted to pH 4 with 0.1 N NaOH and diluted to a total volume of 1.0 mL with water. 3-Phosphoglycerate and dihydroxyacetone phosphate were then separated by HPLC, and the $^3H/^{14}C$ ratio was determined as described above.

Appearance of Solvent Tritium in Remaining Substrate [1-^{14}C]Glyceraldehyde Phosphate and in Product. A mutant isomerase catalyzed reaction was run under conditions similar to those used by Fletcher et al. (1976). The reaction mixture contained (in 1.6 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.5, EDTA (5 mM), NADH (2.2 mg), glycerolphosphate dehydrogenase (200 units), 2 mM (*RS*)-[1-^{14}C]-glyceraldehyde 3-phosphate (0.04 Ci/mol), and tritiated water (27 mCi). After equilibration to 30 °C, two samples (5 μL) were removed and diluted into water (1.0 mL) for later assay to determine the solvent tritium specific radioactivity. A larger sample (200 μL) was also removed and quenched into 1 N HCl (25 μL) and immediately frozen in liquid nitrogen and stored. The isomerase reaction was then initiated in the reaction mixture by the addition of mutant yeast isomerase (0.37 unit). The extent of reaction was monitored at 340 nm with a portion of the reaction mixture (600 μL) in a short-path (2 mm) optical cuvette. At three stages in the reaction, a sample (200 μL) was removed and quenched into 1 N HCl (25 μL). The quenched samples were immediately frozen in liquid nitrogen and stored. The solution in the optical cuvette was also quenched with 1 N HCl (75 μL) and stored. Solvent was then removed from each sample by bulb-to-bulb distillation. The

Table I: Purification of Mutant H95Q Yeast Triosephosphate Isomerase Expressed in *E. coli* Strain DF502(p8)

step	total units	specific catalytic activity (units/mg)	*x*-fold purification	yield (%)
crude lysate	2740	1.5		
ammonium sulfate pellet	2190	4.7	3.2	80
QAE-Sephadex (pH 7.5)	1510	21	16	55
QAE-Sephadex (pH 8.0)	608	53	35	22

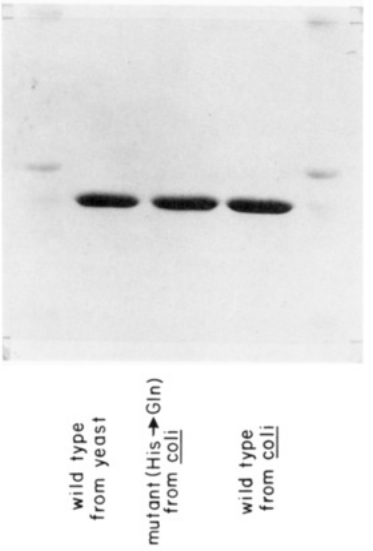


FIGURE 1: Polyacrylamide gel electrophoresis of wild-type and mutant yeast isomerases under denaturing conditions.

sample residues were dissolved in 0.8 mM HCl (0.5 mL), and the solvent was again removed by distillation. This was repeated once. The sample residues were then redissolved in 0.8 mM HCl (0.5 mL) and the enzymes removed by ultrafiltration. The remaining glyceraldehyde phosphate in the samples was then converted into 3-phosphoglycerate. Each sample was neutralized with NaOH (0.1 N); the solution was transferred to an optical cuvette and then diluted with water to a volume of 1.2 mL. NAD^+ (0.1 mg) and sodium arsenate (5 μmol) were added, and the initial absorbance at 340 nm was determined. Isomerase-free glyceraldehyde-3-phosphate dehydrogenase (8 units) was then added, and the reaction was followed to completion by monitoring the absorbance at 340 nm. The glyceraldehyde-3-phosphate dehydrogenase was removed from each sample by ultrafiltration, and the 3-phosphoglycerate and glycerol phosphate were then separated by HPLC, and their specific radioactivity was determined as described above.

RESULTS

The purification of the mutant H95Q[3] yeast isomerase is summarized in Table I. The purified protein gave a single band on polyacrylamide gel electrophoresis under denaturing conditions (Figure 1) and on nondenaturing isoelectric focusing gels (Figure 2). The mutant and wild-type enzymes could be resolved by isoelectric focusing, having p*I* values of 5.60 and 5.61, respectively. Triosephosphate isomerase activity corresponded only to the protein bands on the isoelectric focusing gel, as shown by an isomerase activity stain (data not shown). The molecular weight of the mutant was indistinguishable from that of the wild type, and the sequence of the N-terminus (Ala-Arg-Thr-Phe-) showed no N-terminal methionine.

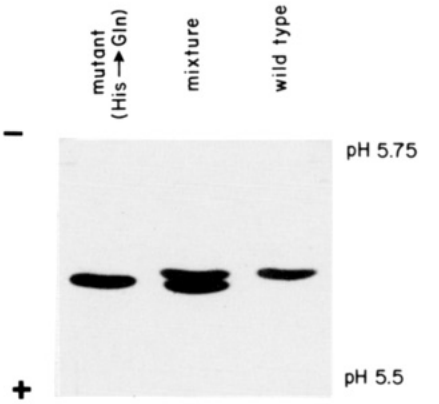


FIGURE 2: Isoelectric focusing gel electrophoresis of wild-type and mutant yeast isomerases under nondenaturing conditions. The gel has been stained for protein.

The steady-state kinetic parameters for the mutant enzyme are shown in Table II. The value of k_{cat} with glyceraldehyde phosphate as substrate was found to be independent of pH over the range 6.5–8.4. All experiments with the mutant were conducted under the same conditions as those with the wild-type yeast isomerase, so that a direct comparison of the results between the two enzymes could be made. The inhibition constants for arsenate (which is a necessary factor in the calculation of K_m^+) and for (*RS*)-glycerol phosphate, 2-phosphoglycolate, and phosphoglycolohydroxamate are also given in Table II.

Dihydroxyacetone phosphate was equilibrated with tritiated solvent by the mutant isomerase to investigate the stereospecificity of proton exchange catalyzed by this enzyme. After equilibration, the dihydroxyacetone phosphate was reisolated and then allowed to react either with chicken muscle triosephosphate isomerase [which is known to catalyze the exchange of only the 1-*pro-R* proton of dihydroxyacetone phosphate: Rieder and Rose (1959)] or with rabbit muscle aldolase [which is known to catalyze the exchange of only the 1-*pro-S* proton: Rieder and Rose (1959) and Rose (1962)]. Incubation with aldolase resulted in negligible release of tritium into solvent, while incubation with chicken triosephosphate isomerase liberated all of the tritium that had been exchanged in by the mutant isomerase, confirming that the mutant labilizes only the 1-*pro-R* proton of dihydroxyacetone phosphate. It was also shown that the mutant enzyme has the same strict stereospecificity with respect to (*R*)-glyceraldehyde phosphate as the wild-type isomerase.

The extent of tritium transfer from [1(*R*)-^{3}H]dihydroxyacetone phosphate to glyceraldehyde phosphate, and hence to the ultimate product 3-phosphoglycerate (expressed as the ratio of the specific radioactivity of the ultimate product *p* and the specific radioactivity of the substrate at the start of the reaction s_0), was investigated in two separate experiments using the mutant yeast isomerase. At 39% reaction, p/s_0 was 0.010 ± 0.001, and at 53% reaction, p/s_0 was 0.008 ± 0.001. The specific radioactivity of the recovered substrate *s* (expressed as a fraction of s_0) at 39% reaction and 53% reaction was found to be 1.16 ± 0.06 and 2.4 ± 0.3, respectively. The tritium transfer experiment was repeated with doubly labeled [1-(*R*)-^{3}H,1-^{14}C]dihydroxyacetone phosphate. The inclusion of a ^{14}C label into the substrate allowed the experimental procedure to be streamlined by eliminating the necessity for en-

[3] We follow here the protocol that, using the single-letter amino acid codes, specifies a mutant as H95Q if His-95 has been changed to Gln.

Table II: Steady-State Parameters for Mutant H95Q Yeast Triosephosphate Isomerase Expressed in *E. coli* DF502(p8) and for Wild-Type Yeast Isomerase Expressed in *E. coli* DF502(p12)[a]

parameter[b]	wild-type isomerase[c]	mutant H95Q isomerase[c]	units	wild type/mutant
k_{cat}^-	$(8.7 \pm 0.3) \times 10^3$	23 ± 1	s^{-1}	380
K_m^-	1.5 ± 0.1	0.7 ± 0.1	mM	1.8
K_m^- (unhydrated)[d]	0.055 ± 0.004	0.027 ± 0.003	mM	1.8
k_{cat}^+	$(7.5 \pm 0.2) \times 10^2$	5.3 ± 0.1	s^{-1}	140
K_m^+	2.3 ± 0.2	3.0 ± 0.1	mM	0.8
K_m^+ (unhydrated)[d]	1.4 ± 0.1	1.77 ± 0.06	mM	0.8
$K_i(HAsO_4^{2-})$	9.6 ± 0.3	15 ± 1	mM	0.6
K_i(glycerol phosphate)	1.4 ± 0.3	1.3 ± 0.2	mM	1
K_i(phosphoglycolohydroxamate)	15 ± 3	530 ± 20	μM	0.028
K_i(phosphoglycolate)	30 ± 1	250 ± 20	μM	0.12
K_{eq}[e]	18 ± 5	18 ± 4	mM	1

[a] Data for the yeast wild-type enzyme are from Nickbarg and Knowles (1988). [b] A (−) as superscript indicates (*R*)-glyceraldehyde 3-phosphate as substrate. A (+) as superscript indicates dihydroxyacetone phosphate as substrate. [c] All experiments were conducted at 30 °C in 66 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (3.3 mM). [d] Values for the unhydrated forms of the substrates; see Albery and Knowles (1976). [e] The overall equilibrium constant determined with the Haldane relationship.

Table III: Variation in the Specific Radioactivity of Substrate [1(*R*)-^{3}H,1-^{14}C]Dihydroxyacetone Phosphate (s) or of Substrate [2-^{3}H,1-^{14}C]Glyceraldehyde Phosphate (p) in the Reactions Catalyzed by the Mutant H95Q Yeast Triosephosphate Isomerase

extent of the reaction of S $(1 - r)$	$^3H/^{14}C$ ratio in the remaining substrate (s)	^3H content in the remaining substrate (s/s_0)
0	4.0 ± 1.1	1.0
0.25	4.7 ± 1.2	1.2
0.46	5.7 ± 0.4	1.4
0.64	8.4 ± 1.0	2.1
0.81	9.2 ± 4	2.3
extent of the reaction of P $(1 - r)$	**$^3H/^{14}C$ ratio in the remaining substrate (p)**	**^3H content in the remaining substrate (p/p_0)**
0	6.8 ± 1.2	1.0
0.21	7.5 ± 1.1	1.1
0.46	9.9 ± 1.7	1.5
0.75	15.5 ± 1.7	2.3

zymatic assay of either the substrate or the product and by allowing the reaction mixture to be rapidly analyzed by HPLC. Determination of the relative specific radioactivities of both the remaining substrate and the product was made from the $^3H/^{14}C$ ratios of the fractions from the HPLC column. A single reaction mixture thus provides data points for several extents of reaction in the time formerly required to obtain data for a single extent of reaction. No enrichment of tritium above background levels was observed in the product 3-phosphoglycerate at any extent of reaction up to 81%. The results for the specific radioactivity of remaining substrate (s/s_0) at various extents of reaction are shown in Table III.

Doubly labeled [2-^{3}H,1-^{14}C]glyceraldehyde 3-phosphate was allowed to react with the mutant isomerase to assess the extent of tritium transfer from substrate to product dihydroxyacetone phosphate in the reverse reaction. The amount of ^3H transferred to the product dihydroxyacetone phosphate (which was converted in situ to glycerol phosphate) was negligible. The specific radioactivity of ^3H in the remaining substrate [1-^{14}C]glyceraldehyde 3-phosphate (which was converted to 3-phosphoglycerate before analysis) is tabulated in Table III.

With dihydroxyacetone phosphate as substrate, exchange–conversion experiments were performed at five extents of reaction for the mutant isomerase. The values obtained for 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 reaction $(1 - r)$, are listed in Table IV and are compared with the results for the wild-type isomerase in Figure 3. The specific radioactivity of tritium in product glyceraldehyde 3-phosphate (which was converted in situ to 3-phosphoglycerate), p (expressed as a fraction of the solvent specific radioactivity, x), was determined and is shown at various extents of reaction in Table IV. To confirm the location of the ^3H label in the product 3-phospho[^{3}H]glycerate, a sample of tritiated 3-

Table IV: Mutant H95Q Isomerase Catalyzed Incorporation of Solvent Tritium into Remaining Substrate Dihydroxyacetone Phosphate and into Product Phosphoglycerate, during the H95Q Mutant Isomerase Catalyzed Reaction of Dihydroxyacetone Phosphate

fractional extent of reaction $(1 - r)$	specific radioactivity of solvent, x (μCi/μmol)	specific radioactivity of remaining substrate, s (μCi/μmol)	specific radioactivity of product, p (μCi/μmol)
0.25	0.165 ± 0.06	0.00335 ± 0.00007	0.0416 ± 0.001
0.51	0.141 ± 0.008	0.0053 ± 0.0003	0.034 ± 0.003
0.75	0.16 ± 0.01	0.018 ± 0.007	0.038 ± 0.007
0.86	0.154 ± 0.005	0.010 ± 0.002	0.037 ± 0.002
0.30[a]	0.199 ± 0.006	0.0026 ± 0.0003	0.044 ± 0.002
0.45[a]	0.199 ± 0.006	0.0057 ± 0.0002	0.049 ± 0.001
0.68[a]	0.199 ± 0.006	0.012 ± 0.001	0.051 ± 0.001
0.53[a]	0.21 ± 0.03	0.0058 ± 0.0002	0.047 ± 0.001
0.66[a]	0.21 ± 0.03	0.0103 ± 0.0006	0.048 ± 0.001
0.73[a]	0.21 ± 0.03	0.013 ± 0.001	0.048 ± 0.001
1.0	0.0966 ± 0.005		0.0222 ± 0.0006

[a] These data were obtained from two separate reaction mixtures with [1-^{14}C]dihydroxyacetone phosphate as the substrate and the double-label HPLC analysis.

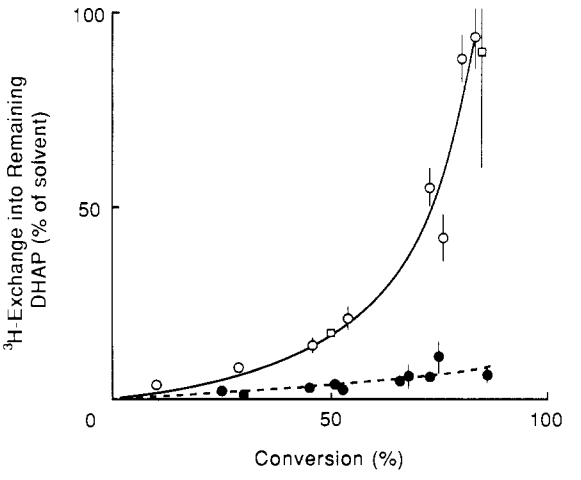


FIGURE 3: Exchange–conversion plot for chicken wild-type (○), yeast wild-type (□), and H95Q yeast mutant (●) isomerases with dihydroxyacetone phosphate as substrate. The data for the chicken wild-type enzyme are from Maister et al. (1976), and the data for the yeast wild-type enzyme and the solid-line fit are from Nickbarg and Knowles (1988). The dashed line is a fit to the mutant data generated with $A_6' = 1.5$, $A_7' = 0.07$, and eq 1 of Maister et al. (1976).

Table V: Mutant H95Q Isomerase Catalyzed Incorporation of Solvent Tritium into Remaining Substrate Glyceraldehyde 3-Phosphate and into Product Glycerol Phosphate, during the H95Q Mutant Isomerase Catalyzed Reaction of Glyceraldehyde 3-Phosphate

fractional extent of reaction $(1 - r)$	specific radioactivity of solvent, x (μCi/μmol)	specific radioactivity of remaining substrate, p (μCi/μmol)	specific radioactivity of product, s (μCi/μmol)
0.37	0.145 ± 0.006	0.0019 ± 0.0003	0.0443 ± 0.0004
0.59	0.159 ± 0.005	0.0032 ± 0.0004	0.050 ± 0.001
0.85	0.158 ± 0.004	0.0038 ± 0.0004	0.039 ± 0.003
0.99	0.13 ± 0.02		0.046 ± 0.003
1.0	0.158 ± 0.003		0.0462 ± 0.0003
0.86[a]	0.164 ± 0.005	0.005 ± 0.001	0.054 ± 0.001
0.95[a]	0.164 ± 0.005		0.056 ± 0.001
1.0[a]	0.164 ± 0.005		0.053 ± 0.001

[a] These data were obtained from a single reaction mixture with [1-^{14}C]glyceraldehyde 3-phosphate as the substrate and the double-label HPLC analysis.

phosphoglycerate was treated with phosphoglycerate mutase and enolase. More than 91% of the tritium was washed out into the solvent. When the same procedure was performed in the absence of phosphoglycerate mutase and enolase, more than 90% of the tritium remained in the nonvolatile residue. Less than 10% of the tritium can therefore be attributed to adventitious labeling on C-3 rather than C-2 of the tritiated 3-phosphoglycerate.

To investigate the unlikely possibility that glyceraldehyde-3-phosphate dehydrogenase catalyzes the exchange of solvent tritium during exchange–conversion experiments using dihydroxyacetone phosphate as substrate, a sample of (*RS*)-glyceraldehyde 3-phosphate was incubated with glyceraldehyde-3-phosphate dehydrogenase in tritiated solvent in the absence of isomerase. This experiment also tests for nonenzymatic incorporation of solvent tritium into glyceraldehyde 3-phosphate. The reaction products, (*S*)-glyceraldehyde 3-phosphate and 3-phosphoglycerate, were separated by DEAE-cellulose chromatography. No significant incorporation of tritium into either product could be detected.

With [1-^{14}C]dihydroxyacetone phosphate as substrate, two exchange–conversion experiments were performed. These experiments provided data for six extents of reaction, plus a reference data point taken before the addition of isomerase. Values of the specific radioactivity of tritium in the remaining substrate dihydroxyacetone phosphate (s) were determined from the ^{3}H/^{14}C ratio of the dihydroxyacetone phosphate peak in the HPLC profile for each sample. This number was multiplied by the value of the specific radioactivity of ^{14}C in dihydroxyacetone phosphate to obtain s (in μCi/μmol), which was then divided by the specific radioactivity of ^{3}H in the solvent, x. The results are tabulated in Table IV and are compared with the results for the wild-type enzyme in Figure 3. Values for the specific radioactivity of tritium in product glyceraldehyde 3-phosphate (converted in situ to 3-phosphoglycerate), p, were determined analogously from the ^{3}H/^{14}C ratio of the HPLC column fraction containing the major portion of 3-phosphoglycerate. The results for the six extents of reaction in Table IV are corrected for the initial amount (3%) of (*R*)-glyceraldehyde 3-phosphate present in the labeled dihydroxyacetone phosphate substrate.

With glyceraldehyde 3-phosphate as substrate, three exchange–conversion experiments were performed with the mutant isomerase. The values of the specific radioactivity of the remaining substrate glyceraldehyde phosphate (which was converted to 3-phosphoglycerate before analysis), p (expressed as a fraction of x), as a function of the extent of reaction, are listed in Table V and are compared with the results obtained with the wild-type enzyme in Figure 4. The specific radioactivity of product dihydroxyacetone phosphate (converted in situ to glycerol phosphate), s (expressed as a fraction of x), is listed in Table V for various extents of reaction. The amount of exchange of solvent tritium with remaining substrate [1-^{14}C]glyceraldehyde 3-phosphate (which was converted to 3-phosphoglycerate before analysis) was determined for one extent of reaction from the ^{3}H/^{14}C ratio of 3-phosphoglycerate. The results are shown in Table V and are compared with the results obtained from the wild-type enzyme in Figure 4. Values for the specific radioactivity of tritium in the ^{14}C-containing product dihydroxyacetone phosphate (which was converted in situ to glycerol phosphate), s, were determined from ^{3}H/^{14}C ratios of the appropriate HPLC column fractions. Values for s were then divided by the specific activity of tritium in the solvent, x. The results for three extents of reaction are shown in Table V.

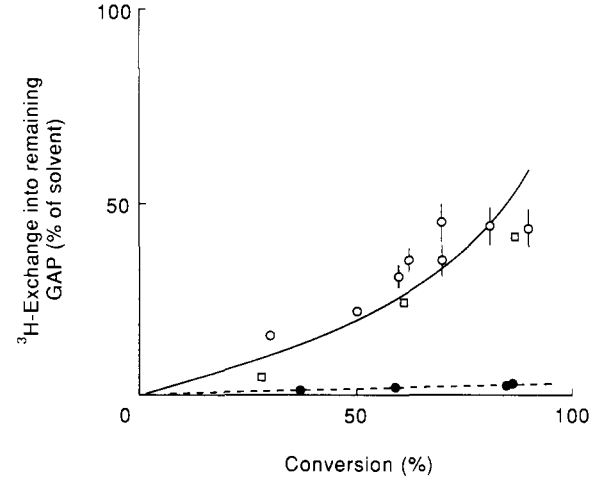


FIGURE 4: Exchange–conversion plot for chicken wild-type (○), yeast wild-type (□), and H95Q yeast mutant (●) isomerases with glyceraldehyde phosphate as substrate. The data for the chicken wild-type enzyme are from Fletcher et al. (1976), and the data for the yeast wild-type enzyme and the solid-line fit are from Nickbarg and Knowles (1988). The dashed line is a fit to the mutant data with $B_6' = 2.2$, $B_7' = 0.04$, and eq 1 of Fletcher et al. (1976).

The kinetic effects of deuterium substitution were investigated with both [2-^{2}H]glyceraldehyde 3-phosphate and [1-(*R*)-^{2}H]dihydroxyacetone phosphate. Comparison of the results generated from the least-squares fitting program ISOVKVC (Cleland, 1979) shows that the primary isotope effects are identical for V and V/K. The deuterium isotope effect on $(k_{cat}^{H})/(k_{cat}^{D})$ is 2.4 ± 0.1 with glyceraldehyde phosphate as substrate and 2.15 ± 0.04 with dihydroxyacetone phosphate as substrate.

Discussion

Although the existence of an electrophilic component in the catalytic mechanism of triosephosphate isomerase is solidly based upon spectroscopic and kinetic observations, and while the crystal structures of the enzymes from both chicken muscle (Banner et al., 1975) and yeast (Alber et al., 1981) have identified His-95 as a likely candidate for the mediation of electrophilic catalysis, the precise mechanistic role of this residue remains unclear. On the one hand, histidine is a potential cationic residue that could stabilize the developing negative charge of the enediolate intermediate, and by proton transfer generate the enediol and a neutral imidazole. The participation of a positively charged histidine side chain is consistent with the pH dependence of the isomerase reaction

(Plaut & Knowles, 1972; Belasco et al., 1978), which indicates that both enzyme and substrate ionizations are important in the physiological pH range. Such studies cannot, however, define the source of the observed pH variation of the steady-state kinetic parameters (Knowles, 1976). On the other hand, there are persuasive structural arguments to suggest that the imidazole ring of His-95 is neutral in the pH range of catalytic activity of the enzyme. Thus the crystal structure of the chicken isomerase positions His-95 at the amino terminus of the α-helix formed by residues 95–103, and the δ-nitrogen of the imidazole side chain is in hydrogen-bonding distance to the main-chain amide nitrogen of Glu-97 (Banner et al., 1975). Similar results are evident from the crystal structures of the native yeast enzyme and its complex with phosphoglycolohydroxamate (R. Davenport, B. Seaton, G. Petsko, and D. Ringe, unpublished results). Protonation of the imidazole group of His-95 would necessitate breaking this putative hydrogen bond, which suggests that this side chain may be neutral in the active enzyme. In this case, the role of His-95 could be to stabilize the charged enediolate by hydrogen bonding from its ϵ-nitrogen (Scheme IB), rather than by actual proton transfer (Scheme IA). The electrophilicity of His-95 might be enhanced by the field induced by the helix dipole formed by residues 97–103 (Petsko et al., 1984). The importance of helix dipoles has been implicated in a variety of contexts (Hol et al., 1981; Hol, 1985), though it has been suggested that solvent screening will reduce the magnitude of the helix dipole effect unless the helix termini are shielded from water (Rogers & Sternberg, 1984), and the functional significance of helix dipoles is difficult to assess. In addition, X-ray crystallography can only locate protons by implication, and the observed structures may not properly reflect the dynamic processes that occur during catalysis in active sites. To examine the role of His-95 (whether protonated or not) in the reaction catalyzed by triosephosphate isomerase, we have analyzed the kinetic and mechanistic behavior of a mutant isomerase in which His-95 has been replaced by Gln.

The wild-type isomerase isolated from *Saccharomyces cerevisiae*, the wild-type yeast isomerase expressed in *E. coli*, and the mutant H95Q yeast isomerase expressed in *E. coli* have all been purified to homogeneity (Figure 1). The mutant enzyme can be separated from the wild-type enzyme by isoelectric focusing under nondenaturing conditions (Figure 2). The mutant enzyme has a slightly lower p*I* than the wild-type enzyme. Analysis of the N-terminal sequence of the mutant and wild-type enzymes shows that this small difference in p*I* does not derive from the presence or absence of N-terminal methionine. The difference in charge of the H95Q mutant is consistent with the replacement of a fully or partially charged histidine (as in Scheme IA) by a neutral glutamine, though the p*I* difference is small and does not require that His-95 be protonated at the pH values where the enzyme is active. When the isoelectric focusing gel is stained by an enzyme activity reagent, the mutant isomerase catalytic activity is seen to comigrate with the single mutant protein band. This observation rules out the possibility that the catalytic activity of the mutant enzyme derives from contamination by wild-type enzyme and demonstrates that the observed catalytic activity is due solely to the mutant protein. This conclusion is supported by the difference in the values of K_m for the mutant and wild-type isomerases. The K_m for (*R*)-glyceraldehyde phosphate in the mutant-catalyzed isomerization is 0.7 ± 0.1 mM, while that with the wild-type enzyme is 1.5 ± 0.1 mM. The value of K_m is a property only of the type of catalytically active molecules, not of their concentration, and a change in the value of K_m indicates a difference in the active enzyme itself.

Steady-State Kinetics. Comparison of the values of k_{cat} for the mutant and wild-type isomerases reveals that a large reduction in catalytic activity has occurred. The k_{cat} for glyceraldehyde 3-phosphate has been reduced by almost 400-fold, while that for dihydroxyacetone phosphate has decreased 140-fold. It seems unlikely that the fall in activity is due to any major structural alteration in the enzyme. Preliminary crystallographic studies show that crystals of the purified mutant protein grown in the presence of phosphoglycolohydroxmate are isomorphous with the corresponding wild-type form, and diffraction data have been collected to 3.0-Å resolution (R. Davenport, F. Hartman, G. Quigley, and G. Petsko, unpublished results; Davenport, 1986). In addition, the K_m values for the substrates are not much perturbed, and the values of K_i for arsenate and (*RS*)-glycerol 3-phosphate are nearly identical for the mutant and wild type. Moreover, the H95Q change leaves the stereospecificity of the catalyzed reaction unaffected, the mutant isomerase showing the same clear stereochemical preferences [for the 1-*pro-R* proton of dihydroxyacetone phosphate and for (*R*)-glyceraldehyde 3-phosphate] as its wild-type progenitor. The sharp reduction in k_{cat} values for the mutant must largely be due to the local modification in active site structure and chemistry caused by the replacement of His-95 by glutamine.

Competitive Inhibitors. Kinetic experiments using substrate analogues expose a further significant difference between the mutant and native enzymes. Thus, although glycerol phosphate and arsenate inhibit the mutant and wild-type isomerases equally, the binding constant of phosphoglycolohydroxamate to the mutant isomerase is 35-fold weaker than that to the wild-type enzyme. Phosphoglycolohydroxamate binds to the wild-type enzyme with a K_i that is invariant between pH 7.5 and pH 8.5 (Collins, 1974), and since the pK_a of the hydroxamic acid moiety is 9.7, this inhibitor presumably binds as the neutral hydroxamate (the phosphate group, as in all bound substrates and inhibitors of the isomerase, is dianionic). The tight binding of phosphoglycolohydroxamate to the native isomerase appears to result from the participation of this substrate isostere in the first step of the normal reaction to yield an enzyme-bound intermediate analogue that is stabilized by His-95. Such stabilization could occur by general acid catalysis or by an electrostatic mechanism, analogous to pathways A and B, respectively, shown in Scheme I. According to these formulations, glycerol phosphate would bind more weakly than phosphoglycolohydroxamate to the native enzyme, being unable to indulge in any partial reactions at the active site. Phosphoglycolate has been shown to bind tightly to the wild-type chicken isomerase (Wolfenden, 1969), and in this case NMR studies have demonstrated that the inhibitor is bound as the trianion (Campbell et al., 1978; Campbell et al., 1979). This behavior is also in accord with the view that 2-phosphoglycolate is an analogue of the reaction intermediate, the binding of which is presumably stabilized by interaction with His-95.

The inhibition constants of the mutant H95Q isomerase support the above arguments. Thus, glycerol phosphate binds as weakly to the mutant as it does to the wild-type enzyme, which is consistent with neither enzyme forming strong interactions with the glycerol phosphate hydroxyl groups. On the other hand, if the tight binding of phosphoglycolohydroxamate results from the enolization of the hydroxamate by the enzyme, then the H95Q mutant should bind phosphoglycolohydroxamate much less tightly than the wild

type, since part of the catalytic apparatus for stabilization of the enediolate (i.e., His-95) is missing. The mutant enzyme would be expected to bind phosphoglycolohydroxamate about as weakly as glycerol phosphate, which is exactly what is found (Table II). Similar weak binding to the mutant enzyme is predicted for phosphoglycolate, and the dissociation constant for this inhibitor is, in fact, nearly as high as that for phosphoglycolohydroxamate. These results suggest that the reason for the sharp reduction in k_{cat} values for the H95Q mutant derives from the inability of the mutant to stabilize or neutralize the negatively charged enediolate oxygen.

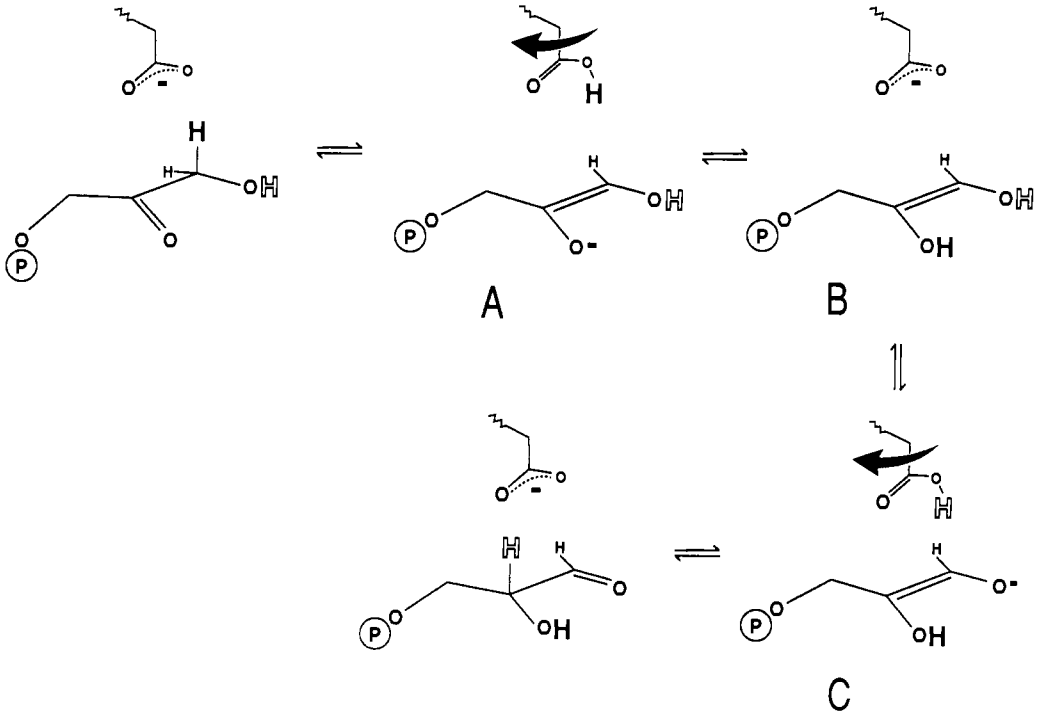
Isotope Partitioning Experiments. The delineation of the energetics of triosephosphate isomerase requires the blending of a variety of experiments in which the partitioning of the various forms of the liganded enzyme is defined by experiments that trace the course of both substrate-derived and solvent-derived tritium labels. When [1(*R*)-^{3}H]dihydroxyacetone phosphate is the substrate, the label may be found in the product glyceraldehyde phosphate (which is converted in situ to 3-phosphoglycerate), it may be exchanged out into the solvent, or it may remain in place in the shrinking pool of remaining dihydroxyacetone phosphate molecules. The presence of tritium at C-2 of the product glyceraldehyde phosphate would represent a proton *transfer* from the C-1 of the substrate dihydroxyacetone phosphate. The amount of transfer of label with the mutant isomerase is less than 1% (measured at two extents of reaction), and the new proton that becomes attached to C-2 of the product derives almost entirely from the solvent. [In comparison, the amount of transfer observed with the wild-type yeast isomerase is about 3% at 66% of reaction (Nickbarg & Knowles, 1988).] The tritium content of the remaining substrate pool is also informative, and the specific radioactivity of the remaining [1(*R*)-^{3}H]dihydroxyacetone phosphate depends upon (a) the primary tritium isotope effect (which tends to increase the specific radioactivity of the remaining substrate by preferentially consuming unlabeled molecules) and (b) the partitioning of the enzyme-bound enediol forward to product and back to substrate (where a high proportion of backreaction will wash out isotope into solvent and decrease the specific radioactivity of the remaining substrate). In fact, the remaining substrate is somewhat enriched in tritium in the course of the enzymatic conversion (2.3-fold after 81% of reaction), showing that the primary isotope effect dominates these processes. Paralleling these results, a tritium transfer experiment in the reverse direction using [2-^{3}H,1-^{14}C]glyceraldehyde phosphate as substrate showed no significant transfer of tritium to the product dihydroxyacetone phosphate, while the tritium content of the remaining glyceraldehyde phosphate was enriched by more than 2-fold after 75% of reaction. In our experiments with the wild-type enzymes from chicken and from yeast, the small amounts of tritium transfer from substrate to product indicated the existence of an enzyme-bound intermediate from which the abstracted substrate proton exchanges rapidly with the solvent protons (Herlihy et al., 1976; Nickbarg & Knowles, 1988). An even smaller amount of tritium transfer is now observed with the H95Q mutant isomerase. The implications of these results are discussed below.

When the mutant isomerase catalyzed reaction is run in tritiated solvent with unlabeled dihydroxyacetone phosphate as substrate, little tritium is incorporated back into the pool of remaining substrate even after most of the substrate has been consumed (see Figure 3). Since the intermediate *appears to be* in rapid exchange with the solvent (see above), the absence of solvent tritium incorporation into remaining substrate would suggest that the intermediate partitions forward to product glyceraldehyde phosphate. If this were the case, then the opposite extreme should be observed when the reaction is run in the other direction, with glyceraldehyde phosphate as substrate. That is, if the reaction intermediate partitions preferentially toward glyceraldehyde phosphate, then when the reaction is run with glyceraldehyde phosphate as substrate, its C-2 proton should effectively *preequilibrate* with the labeled solvent protons, and the specific radioactivity of tritium in the remaining substrate should rise rapidly to a value near to that of the solvent. However, as is clear from Figure 4, very little solvent tritium is incorporated into the remaining substrate glyceraldehyde phosphate over the course of the mutant isomerase catalyzed conversion to dihydroxyacetone phosphate. Given a mechanism of isomerase catalysis that involves proton abstraction and exchange by a single enzyme base, these two sets of results seem to be mutually incompatible. That is, *both* of the exchange–conversion experiments suggest that the intermediate partitions forward, which obviously cannot occur in both directions of reaction. [The absence of solvent tritium in remaining substrate when the reaction is run in either direction could, of course, derive from the trivial reason that the isomerase was not completely removed before the substrate was taken up in unlabeled solvent and that, in this milieu, all tritium was washed out. Control experiments were therefore carried out to show that the mutant enzyme is catalytically inactive below pH 3 and that the pH of the quenched reaction remains below 2 until all traces of isomerase have been removed. The experimental procedures were, moreover, identical for the mutant enzyme (where essentially no tritium was found in remaining substrate) and the wild-type enzyme [where significant labeling of remaining substrate was observed: Nickbarg and Knowles (1988)]. Finally, the finding of isotopic *enrichment* in remaining substrate when labeled starting material is used confirms the validity of the quenching method and analytical procedures described.]

In principle, the observed lack of incorporation of solvent tritium into remaining substrate in both the forward reaction and the reverse reaction might result from the existence of very large primary tritium isotope effects that discriminate against tritium in the proton transfer steps both preceding and following the intermediate. Large isotope effects on both proton transfer steps would discriminate against tritium entering either substrate or product. We know, however, from the discrimination against solvent tritium in the formation of product, that the tritium isotope effects in these proton transfer steps are *not* very large. Thus when dihydroxyacetone phosphate is the substrate, the discrimination against tritium incorporation into the product is 4.2-fold, and when glyceraldehyde phosphate is the substrate, the discrimination against tritium in the product is 3-fold. These results are independently confirmed by the values of the kinetic deuterium isotope effects.[4] The deuterium isotope effect on k_{cat} with [1(*R*)-^{2}H]dihydroxyacetone phosphate is 2.15 ± 0.04 [equivalent to 3.0 for tritium using the Swain–Schaad relationship: Swain et al. (1958)],

[4] Tritium isotope effects are necessarily determined in competitive experiments, in which unlabeled molecules compete with tritiated molecules to illuminate transition states that may not be rate determining in the overall reaction sequence, whereas deuterium isotope effects are normally determined noncompetitively and provide information about the kinetic importance of protonation and deprotonation steps in the overall reaction. For the H95Q mutant isomerase, the substrate enolization steps have been slowed down, and neither enolization step can be cleanly rate limiting, since significant isotope effects are seen for the reaction of each of the deuteriated substrates. The isotope effects that are observed do not therefore reflect the intrinsic effects.

Scheme II: Proposed Mechanism of the H95Q Mutant Yeast Isomerase[a]



[a] Glu-165 slowly abstracts the 1-*pro-R* proton of dihydroxyacetone phosphate to give the enediolate A. This species collapses rapidly to give the bound enediol B. The carboxylate of Glu-165 can then abstract the other oxygen-bound proton to yield C, collapse of which gives the final product (*R*)-glyceraldehyde 3-phosphate.

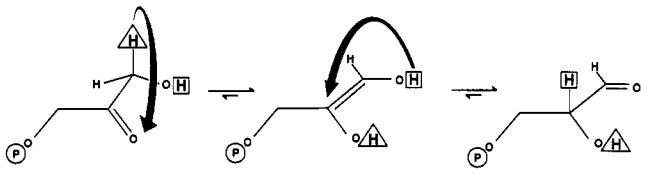
in excellent agreement with the analogous tritium discrimination value of 3.0 ± 0.3 obtained with glyceraldehyde phosphate as substrate in tritiated water. The deuterium kinetic isotope effect obtained with [2-^{2}H]glyceraldehyde phosphate as substrate is 2.4 ± 0.1 (equivalent to 3.5 for tritium), which is reasonably consistent with the tritium discrimination value of 4.2 ± 0.2 obtained with dihydroxyacetone phosphate as substrate in tritiated solvent. Even though these isotope effects are not intrinsic, they are not large enough to resolve the dilemma posed by the two exchange–conversion experiments described above. Solvent tritium enters the remaining substrate very much more slowly than the substrate is converted to product, *irrespective* of the direction in which the reaction is run.

An alternative pathway that could account for the observed lack of exchange of the remaining substrate with tritiated solvent would be a two-base mechanism, such as has been proposed for proline racemase (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975; Albery & Knowles, 1986) and for methylmalonyl-CoA epimerase (Fuller & Leadlay, 1983; Leadlay & Fuller, 1983). These enzymes are believed to use one active site base to abstract a proton from the substrate, and the intermediate anion thus formed is then reprotonated by a second catalytic group to form the product. If the enzymic catalytic acids are sequestered during catalysis and can only exchange protons with the solvent at the free enzyme stage, then no equilibration of solvent tritium into the remaining substrate will be observed in either the forward or the reverse reaction. In addition, since the enzyme-bound intermediate is reprotonated by a second enzymic acid, no transfer of tritium from initially labeled substrate to product will be seen. Normal tritium discrimination values are likely, since the new proton in the product is derived from the second enzymic base which equilibrates with solvent protons at the free enzyme stage. Such a two-base mechanism is, however, most unlikely for our mutant triosephosphate isomerase. Crystallographic evidence from the isomerase complex with phosphoglycolohydroxamate shows that only a single base, Glu-165, is positioned to mediate proton transfer processes with the proper stereospecificity (R. Davenport, B. Seaton, G. Petsko, and D. Ringe, unpublished results). It is, moreover, difficult to imagine how *removal* of a histidine residue (in the H95Q mutant) would change the mechanism from a single-base pathway (in the wild-type) into one that uses two enzymic bases, while maintaining the original stereospecificity of proton transfer. As will be seen below, the experimental results are more easily accommodated by a similar and more economical hypothesis.

The features of the H95Q mutant isomerase that must be considered are the decreased ability of the mutant enzyme to stabilize the bound reaction intermediate (as reflected by the weaker binding of its analogues phosphoglycolohydroxamate and phosphoglycolate) or to catalyze the proton transfer reaction (as shown by the lowered values of k_{cat}). In addition, the proton exchange reactions with solvent have been altered so that, while no transfer of tritium from labeled substrate to product is detectable in either direction (i.e., there is *complete loss* of substrate tritium to the medium), no solvent-derived tritium appears in either substrate (i.e., there is *no gain* of tritium from the medium into remaining substrate). These results present a paradox. While the tritium transfer experiments indicate that the proton abstracted from substrate equilibrates rapidly with the solvent, the exchange–conversion experiments seem inconsistent with any such isotopic equilibrium. The mechanistic pathway followed by the enzyme has evidently been altered by the H95Q mutation.

The New Mechanistic Pathway. The catalytic consequences of the H95Q mutation may be more clearly appreciated if we consider the role of His-95 in the mechanism of the wild-type enzyme. As shown in Scheme II, the enzyme binds the substrate in an extended conformation with the hydroxyl and carbonyl oxygens coplanar with each other and with the

Scheme III: Summary of the Proposed Proton Transfer Processes in the H95Q Mutant



phospho group. This cisoid configuration is consistent with the stereospecificity of proton transfer (Reider & Rose, 1959), and with the attack of sodium borohydride on the bound substrate which is known to occur on the *si* face of the substrate's carbonyl group (Webb & Knowles, 1974). The placement of the phospho moiety is logical for stereoelectronic reasons, since a coplanar orientation will disfavor the elimination of inorganic phosphate from the enediol intermediate (Alber et al., 1981; Richard, 1984). Once bound, the abstraction of the 1-*pro-R* proton of dihydroxyacetone phosphate by the γ-carboxylate of Glu-165 would be facilitated by electrophilic assistance from His-95. As will become apparent below, the assignment of this role to His-95 allows us nicely to explain the behavior of the H95Q mutant.

From the results presented in Table II, it appears that the H95Q mutant binds substrate and substrate analogues in the same fashion as the wild-type enzyme. Glu-165 still abstracts the *pro-R* proton of dihydroxyacetone phosphate, but without the catalytic assistance provided by histidine, and we therefore expect that the mutant enzyme will catalyze the enolization less effectively, to give a less stable enediol*ate* in the active site (A in Scheme II). What are the possible fates of this intermediate? First, the enediolate can simply reprotonate on carbon and re-form the starting material. Second, since the enediolate is a strong base and the γ-COOH of Glu-165 is a nearby acid,[5] the enediolate might reprotonate *on oxygen* to form the enzyme-bound enediol (B in Scheme II). This complex has the neutral enediol lying below the carboxylate of Glu-165. To continue toward product, the enzyme must abstract the other oxygen-bound proton of the enediol (using its only base, Glu-165), and the resulting carboxyl group in C can then protonate the enediolate on C-2 to yield product glyceraldehyde phosphate. This pathway allows Glu-165, from its position above the plane of the enediol, to mediate two types of proton shuttle between carbon and oxygen. The first involves abstraction of the 1-*pro-R* proton of dihydroxyacetone phosphate and delivery of this proton to the C-2 carbonyl oxygen, and the second involves abstraction of the C-2 proton of glyceraldehyde 3-phosphate and its delivery to the C-1 carbonyl oxygen (see Scheme III). This scheme does accommodate all the differences, both qualitative and quantitative, that we have observed between the wild-type and the mutant isomerases.

First, little if any transfer of tritium from labeled substrate to product is expected from this mechanism, since the proton abstracted from carbon by Glu-165 is itself captured by the enediolate oxygen and the new carbon-bound proton of the product derives from the other hydroxyl group of the enediol intermediate, which equilibrated with solvent before the substrate bound to the active site. Second, when the reaction is conducted in tritiated solvent, the specific radioactivity of the product will reflect that of the solvent, modulated by the size of the primary tritium isotope effect that occurs in the proton transfer step. This accommodates both the appearance of tritium in the product and the existence of a significant discrimination against tritium in the product formed in either of the reaction directions. Third, significant levels of solvent isotope are expected *not* to appear in the remaining substrate in either direction, provided that the carboxyl group of Glu-165 that is transiently protonated (in A and C) en route to the enzyme-bound intermediate complex (B) does not exchange its proton with those of the solvent. This behavior is expected, since exchange would require that solvent water could compete for the proton on Glu-165 with the very much stronger base, the unstabilized enediolate. In the wild-type enzyme, the proton of the carboxyl group of Glu-165 is lost either to water or to a carbon center of the enzyme-bound intermediate: the former process is favored, and complete proton exchange is seen. For the mutant enzyme, water and a naked enediolate oxygen compete for the proton on the carboxyl group of Glu-165. The enediolate obviously prevails and thus prevents the exchange reaction between water and carboxyl group of Glu-165 from equilibrating the proton abstracted from the substrate with those of solvent.

The results reported in this paper, while requiring a new mechanistic pathway of the kind outlined above, do not allow us to differentiate between the mechanisms represented in Scheme I. If His-95 behaves merely as a hydrogen-bond donor as in Scheme IB, substitution by glutamine might not be expected to produce a severe reduction in catalytic activity, since the side chain of glutamine is reasonably similar in polarity and hydrogen-bonding capability to that of histidine. If, on the other hand, the imidazolium group of His-95 acts as a proton donor, then replacement by glutamine would be expected to have relatively grave catalytic consequences. These alternatives can only be resolved by an experimental determination of the pK_a of His-95 in the wild-type enzyme, for which purpose the existence of the H95Q mutant will prove invaluable.

In summary, analysis of the H95Q yeast isomerase mutant has shown us that the catalytic mechanism for triosephosphate isomerase is more finely tuned and less resistant to perturbation than might have been thought. Substitution of Gln for His at the active site no longer produces a favorable environment for the formation of an enediol intermediate. Indeed, the removal of this electrophile both drastically slows the enolization steps and also forces a change in catalytic mechanism. These effects emphasize the fact that detailed mechanistic analysis may routinely be necessary if we are properly to appreciate the consequences of site-directed mutations at enzyme active sites.

[5] It is already known from the crystal structure of the enzyme–substrate complex that the carboxylate oxygens of Glu-165 are nicely positioned to abstract either the *pro-R* proton of dihydroxyacetone phosphate or the C-2 proton of glyceraldehyde phosphate, and in this position little more than a single bond rotation of the side chain of Glu-165 would be required for the carboxyl group of this residue to reprotonate the enediolate on *oxygen*.

ACKNOWLEDGMENTS

We are especially grateful to J. D. Hermes for his helpful technical and scientific advice during the course of this work.

Registry No. His, 71-00-1; Gln, 56-85-9; GAP, 591-57-1; DHAP, 57-04-5; PGH, 51528-59-7; $HAsO_4^{2-}$, 16844-87-4; glycerol phosphate, 17989-41-2; phosphoglycolate, 13147-57-4; 3-phospho[2-^{3}H]glycerate, 114978-74-4; [1-^{14}C]glyceraldehyde phosphate, 114978-75-5; [2-^{3}H,1-^{14}C]glyceraldehyde phosphate, 114978-76-6; 3-phospho[1-^{14}C]glycerate, 23520-16-3; [1-^{14}C]dihydroxyacetone phosphate, 23520-17-4; triosephosphate isomerase, 9023-78-3.

REFERENCES

Addadi, L., Jaffe, E. K., & Knowles, J. R. (1983) *Biochemistry 22*, 4494–4501.

Alber, T., & Kawasaki, G. (1982) *J. Mol. Appl. Genet. 1*, 419–434.
Alber, T., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Rivers, P. S., & Wilson, I. A. (1981) *Philos. Trans. R. Soc. London, B 293*, 159–171.
Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5627–5631.
Albery, W. J., & Knowles, J. R. (1986) *Biochemistry 25*, 2572–2577.
Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. L., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. D., Priddle, J. D., & Waley, S. G. (1975) *Nature (London) 255*, 609–614.
Belasco, J. G., & Knowles, J. R. (1980) *Biochemistry 19*, 472–477.
Belasco, J. G., Herlihy, J. M., & Knowles, J. R. (1978) *Biochemistry 17*, 2971–2978.
Browne, C. A., Campbell, I. D., Kiener, P. A., Phillips, D. C., Waley, S. G., & Wilson, I. A. (1976) *J. Mol. Biol. 100*, 319–343.
Campbell, I. D., Jones, R. B., Kiener, P. A., Richards, E., Waley, S. G., & Wolfenden, R. (1978) *Biochem. Biophys. Res. Commun. 83*, 347–352.
Campbell, I. D., Jones, R. B., Kiener, P. A., & Waley, S. G. (1979) *Biochem. J. 179*, 607–621.
Cardinale, G. J., & Abeles, R. H. (1968) *Biochemistry 7*, 3970–3978.
Casal, J. I., Ahern, T. J., Davenport, R. C., Petsko, G. A., & Klibanov, A. M. (1987) *Biochemistry 26*, 1258–1264.
Chen, T. Y., & Toribara, W. H. (1956) *Anal. Chem. 28*, 1756–1758.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Collins, K. D. (1974) *J. Biol. Chem. 249*, 136–142.
Davenport, R. C., Jr. (1986) Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.
de la Mare, S., Coulson, A. F. W., Knowles, J. R., Priddle, J. D., & Offord, R. E. (1972) *Biochem. J. 129*, 321–331.
Fletcher, S. J., Herlihy, J. M., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5612–5617.
Fuller, J. Q., & Leadlay, P. F. (1983) *Biochem. J. 213*, 643–650.
Guilford, W. J., Copley, S. D., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5013–5019.
Hall, A., & Knowles, J. R. (1975) *Biochemistry 14*, 4348–4352.
Hegarty, G. G., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 7188–7189.
Herlihy, J. M., Maister, S. G., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5607–5612.
Hol, W. G. J. (1985) *Prog. Biophys. Mol. Biol. 45*, 149–195.
Hol, W. G. J., Halie, L. M., & Sander, C. (1981) *Nature (London) 294*, 532–536.
Knowles, J. R. (1976) *Crit. Rev. Biochem. 4*, 165–173.
Leadlay, P. F., & Fuller, J. Q. (1983) *Biochem. J. 213*, 635–642.
Leadlay, P. F., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5617–5620.
Maister, S. G., Pett, C. P., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5607–5612.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Messing, J. (1983) *Methods Enzymol. 101*, 20–78.
Nickbarg, E., & Knowles, J. R. (1988) *Biochemistry* (preceding paper in this issue).
Norris, K., Norris, F., Christianson, L., & Fiil, N. (1983) *Nucleic Acids Res. 11*, 5103–5112.
Petsko, G. A., Davenport, R. C., Fraenkel, D., & Rajbhandary, U. L. (1984) *Biochem. Soc. Trans. 12*, 229–232.
Plaut, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 311–320.
Raines, R. T., Sutton, E. L., Straus, D. R., Gilbert, W., & Knowles, J. R. (1986) *Biochemistry 25*, 7142–7154.
Richard, J. P. (1984) *J. Am. Chem. Soc. 106*, 4926–4936.
Rieder, S. V., & Rose, I. A. (1959) *J. Biol. Chem. 234*, 1007–1010.
Rogers, N. K., & Sternberg, M. J. E. (1984) *J. Mol. Biol. 174*, 527–542.
Rose, I. A. (1962) *Brookhaven Symp. Biol. 15*, 293–309.
Rudnick, G., & Abeles, R. H. (1975) *Biochemistry 14*, 4515–4522.
Scopes, R. K. (1968) *Biochem. J. 107*, 139–150.
Straus, D., & Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2014–2018.
Straus, D., Raines, R., Kawashima, E., Knowles, J. R., & Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2272–2276.
Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885–5893.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259–268.
Waley, S. G., Miller, J. C., Rose, I. A., & O'Connell, E. L. (1970) *Nature (London) 227*, 181.
Webb, M. R., & Knowles, J. R. (1974) *Biochem. J. 141*, 589–592.
Webb, M. R., & Knowles, J. R. (1975) *Biochemistry 14*, 4692–4698.
Wolfenden, R. (1969) *Nature (London) 223*, 704–705.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.