

The Role of Water in the Catalytic Efficiency of Triosephosphate Isomerase^{†,‡}

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ABSTRACT: The structural basis for the effect of the S96P mutation in chicken triosephosphate isomerase (cTIM) has been analyzed using a combination of X-ray crystallography and Fourier transform infrared spectroscopy. The X-ray structure is that of the enzyme complexed with phosphoglycolohydroxamate (PGH), an intermediate analogue, solved at a resolution of 1.9 Å. The S96P mutation was identified as a second-site revertant when catalytically crippled mutants, E165D and H95N, were subjected to random mutagenesis. The presence of the second mutation leads to enhanced activity over the single mutation. However, the effect of the S96P mutation alone is to decrease the catalytic efficiency of the enzyme. The crystal structures of the S96P double mutants show that this bulky proline side chain alters the water structure within the active-site cavity (E165D; ref 1) and prevents nonproductive binding conformations of the substrate (H95N; ref 2). Comparison of the S96P single mutant structure with those of the wild-type cTIM, those of the single mutants (E165D and H95N), and those of the double mutants (E165D/S96P and H95N/S96P) begins to address the role of the conserved serine residue at this position. The results indicate that the residue positions the catalytic base E165 optimally for polarization of the substrate carbonyl, thereby aiding in proton abstraction. In addition, this residue is involved in positioning critical water molecules, thereby affecting the way in which water structure influences activity.

Triosephosphate isomerase (TIM, EC 3.2.1.1) catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP). Extensive studies of the yeast and chicken muscle enzymes have established that the mechanism of the isomerization is base-catalyzed proton transfer with electrophilic and/or general acid assistance (ref 1, Figure 1). The reaction rate is limited by the rate of diffusion of substrate onto and off of the enzyme surface (3), and thus TIM is said to have evolved to catalytic perfection (4, 5). Many experimental techniques have been applied to TIM in order to establish its catalytic mechanism and the way in which the structure of the enzyme determines the mechanism. We have utilized two such techniques in the work presented here, both of which have been applied previously to wild-type TIM as well as to several mutants.

X-ray crystallography of the complex of TIM with the inhibitor phosphoglycolohydroxamate (PGH), an analogue of the intermediate in the catalytic reaction (6), is thought to give the best approximation of the structure of the enzyme in its catalytically competent form (7, 8). Fourier transform infrared spectroscopy (FTIR) has allowed the observation of the state of the carbonyl groups of the substrates when bound to the enzyme and has proven useful in determining the degree to which the enzyme polarizes these carbonyl groups toward the transition state of the reaction (9, 10).

Triosephosphate isomerase has several conserved active-site residues responsible for catalysis, including E165, the catalytic base, which is positioned above the substrate in the active site; H95, the residue that appears to be responsible for shuttling protons between the two oxygens of the enediolate intermediates in the reaction; and K13, which is responsible for creating a positively charged environment in the active site so that the phosphodianion substrate can bind (refs 8, 11; Figure 2.). Serine 96 is also present in the active site, although the side chain of this residue is in a different position in the substrate-free isomerase structure compared to the structure of the isomerase-PGH complex (12, 11, 13). In the substrate-free form of the enzyme, the side chain of E165 is rotated away from the position where substrate binds, and its carboxylate group is hydrogen-bonded to the hydroxyl of S96. In the inhibitor-bound form, the side chain of E165 is pointing toward the O1-C-C(N)-O2 plane of the substrate analogue PGH, and the carboxylate

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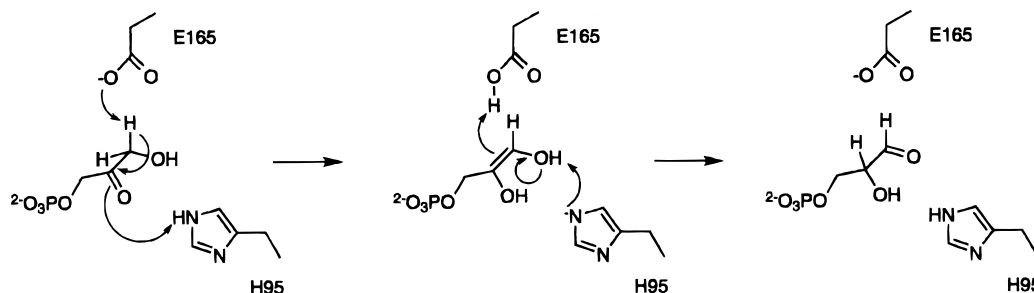


FIGURE 1: Reaction catalyzed by triosephosphate isomerase. The important catalytic residues are E165 (the catalytic base), H95 (the proton shuttle), and K13.

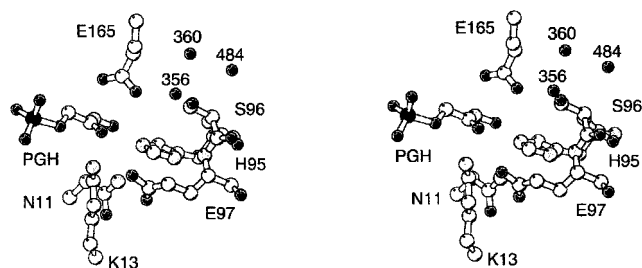


FIGURE 2: Stereo figure of the active site of triosephosphate isomerase showing the relative positions of the active-site residues and waters. The figure was prepared using MOLSCRIPT (43).

group is no longer hydrogen-bonded to the serine, but is instead perfectly positioned to abstract a proton from the substrate. The serine residue in the PGH-bound form is rotated out toward the solvent and interacts with a water molecule and the side chain of histidine 110. Thus, serine 96 seems to play a role in positioning the catalytic base E165 and possibly in influencing the pK_a of that base.

The roles of the two active-site residues, E165 and H95, have been probed by mutagenesis (9, 14, 15). The importance of the position of the catalytic base relative to the substrate was explored by changing the glutamic acid to aspartic acid (E165D), thereby shortening the distance between them by approximately 1 Å. The mutant isomerase has a catalytic efficiency (k_{cat}/K_m) that is reduced 300-fold compared to that of the wild-type isomerase (14). The importance of histidine acting as a proton shuttle between the two oxygen atoms of the two enediolate intermediates in the reaction and its role in polarizing the substrate carbonyl groups were addressed by changing this residue to glutamine and asparagine (10, 15, 16). Glutamine places an NH group at the position of the epsilon nitrogen of histidine, whereas asparagine places the same group at the delta nitrogen position. In both cases, although a hydrogen-bonding capability is still available, proton transfer is not possible. The catalytic efficiency of the H95N mutant is reduced by 3000-fold relative to that of the wild-type. The H95Q mutant is similarly crippled and also appears to undergo a subtle change in mechanism.

Because TIM seems to have evolved to catalytic perfection, an attempt was made to take catalytically damaged mutants and chart an alternate pathway back to maximum efficiency by random mutagenesis. The genes encoding each of two mutants, E165D and H95N, were subjected to random mutagenesis, and transformants that synthesize isomerases with increased catalytic activity were isolated by selection. Surprisingly, the same second-site suppressor locus partially corrects each lesion. The catalytic potency of both the E165D mutant isomerase (by 20-fold) and the H95N mutant

isomerase (by 60-fold) improved with the same second-site alteration, serine 96 to proline (S96P) (17, 18).

Both spectroscopic and structural analyses of the E165D and E165D/S96P mutants indicate that the loss of activity in the single mutant is due to a loss of proximity between the carboxylate group and the substrate. In the pseudorevertant this loss of proximity is partly overcome by better balancing the pK_a 's of the catalytic base and the substrate. This adjustment is accomplished by altering the number and positions of bound water molecules in the active site, resulting in better ground-state stabilization of the first intermediate in the pathway, the enediolate (1).

Spectroscopic and structural analyses of the H95N and H95N/S96P mutants indicate that the loss of activity in the single mutant lies in its inability to bind substrate in the planar, cisoid conformation, the conformation required for efficient isomerization. In the double mutant this problem is partly corrected by the presence of the proline residue. In addition, there is a significant rearrangement of water molecules in the active-site cavity between the single and double mutants (2).

Since a proline in place of serine at position 96 has the ability to improve the catalytic efficiency of several active-site crippled mutants, the role of this residue alone on the catalytic activity was studied. Changing serine 96 to proline in the wild-type enzyme reduces k_{cat}/K_m by 20-fold. This surprising observation raises fundamental questions concerning the role of this conserved side chain in the catalytic activity of wild-type TIM. The experiments reported here show that the effect of the S96P mutation is primarily on the organization of water molecules in the active site of the isomerase.

MATERIALS AND METHODS

Reagents. DL-Glyceraldehyde 3-phosphate diethyl acetal, glucuronolactone, histidine, streptomycin, ampicillin, reduced nicotinamide adeninedinucleotide (NADH), ethylenediamine-tetraacetic acid (EDTA) disodium salt, Dowex-50W (H⁺ form), and QAE Sphadex A-120 were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) 8000 was obtained from Sigma Chemical Co. (ST. Louis, MO). Poly(ethylene glycol) 8000 was obtained from US Biochemicals (Cleveland, OH). DL-Glyceraldehyde 3-phosphate diethyl acetal was deprotected to DL-glyceraldehyde 3-phosphate according to the manufacturer's instructions, but using one-tenth of the volume of H₂O so that the resulting solution was 10 times as concentrated. Phosphoglycolohydroxamate was prepared by J. Belasco according to the procedure of Collins (6). Bromohydroxyacetone phosphate (BHAP) was

prepared as described by de la Mare et al. (19). Glycerol-3-phosphate dehydrogenase was obtained from Boehringer-Mannheim and was made free of TIM activity by Centricon dialysis of a 1 mL sample of the protein against 100 mM triethanolamine-HCl, pH 7.6, 1 mM EDTA. The dialyzed protein was then treated with a 100-fold molar excess of BHAP. Oligonucleotides were prepared on a Milligen 7500 DNA synthesizer according to the manufacturer's protocols and were not further purified before use. All other reagents were from commercial sources and were used without further purification. Specifically ^{13}C -labeled substrates used in the FTIR experiments were prepared as described in Komives et al. (10).

Protein Production. The gene for the mutant was prepared by mutagenesis of the gene for the wild-type TIM from chicken muscle contained in a phagemid vector that was a derivative of pBS (\pm) and has been described (18). This phagemid vector allowed the efficient production of single-stranded DNA for mutagenesis. The mutant in which Ser 96 was changed to proline (S96P) was constructed by using an oligonucleotide-directed mutagenesis kit (Amersham, Arlington Height, IL) and a mutagenic primer having the sequence 5'-CTG GGC CAC C(T in wild-type)CA GAG CGG-3' following the method of Eckstein (20). The gene for the mutant was further subcloned into the high-expression vector pKK 223-3 (Pharmacia, Piscataway, NJ) to avoid instabilities of the phagemid upon large-scale growth. The phagemid contains, on an *EcoRI* to *PstI* fragment, the *trc* promoter upstream from the complete TIM gene and allowed the production of 50–80 mg of protein per liter of cells. The expression vectors were used to transform *Escherichia coli* strain DF502, which is a strepR, tpi strain that was kindly provided by D. Fraenkel and has been previously described (21).

Large amounts of pure protein were prepared by growing the bacterial transformants in a final volume of 10 L of M63 salts (22) containing casamino acids (0.5% w/v), glucuronolactone (0.4% w/v), glycerol (0.1% w/v), MgSO_4 (1 mM), thiamine (1 mM), L-histidine (80 mg/L), streptomycin (100 mg/L), and ampicillin (200 mg/L). Cells were harvested after 12–20 h by centrifugation at 3000g. The cells were lysed in a continuous flow French pressure cell (Aminco, Urbana, IL), and lysate was centrifuged at 8500g for 1 h to remove cell debris. The ammonium sulfate fraction from 55% to 90% saturation was collected and dialyzed against TE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA) overnight. The following day, the crude protein was loaded onto a 300 mL column of QAE Sephadex A-120 equilibrated with TE buffer and eluted with a linear gradient of 0–300 mM KCl (1 L to 1 L). The protein was finally purified on a MonoQ 10/10 column using the same gradient. Purity of the protein was assessed by silver staining overloaded 15% SDS-PAGE gels (23). Concentration of the protein was achieved by centriprep and Centricon concentration (Amicon, Danvers, MA). The purified protein was assayed for conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate according to the method of Putman et al. (24), and k_{cat} and K_m values obtained were identical to those previously reported. Assays were conducted at 30 °C in a solution containing 0.1 M triethanolamine, buffered at pH 7.6, and 10 mM EDTA.

Protein Crystallization. The purified protein was dialyzed into MilliQ H_2O and concentrated to 20 mg/mL (calculated

Table 1: Statistics of Data Collection and Refinement for the S96P Mutant Isomerase Structure

data collection	
space group	$P2_12_12_1$
unit cell dimensions	$a = 136.1 \text{ \AA}$ $b = 74.3 \text{ \AA}$ $c = 53.5 \text{ \AA}$
resolution limit	1.94 \AA
no. of reflections (total; $I/\sigma(I) > 0$)	70 742
no. of reflections (unique; $I/\sigma(I) > 0$)	33 134
R_{sym} (% on I) ^a	10.1
completeness (% overall)	81
refinement	
resolution range	6.0–1.94 \AA
no. of reflections ($I/\sigma(I) > 0$)	31 892
final R -factor ^b	20.2%
no. of protein atoms	3714
no. of inhibitor atoms	20
no. of waters	281
rms deviations	
bond distances (\AA)	0.016
bond angles (deg)	3.0
B -factor model	individual

^a $R_{\text{sym}} = \sum (|I(h,j) - I(h)|) / \sum I(h,j)$, where $I(h,j)$ are symmetry-related intensity observations and $I(h)$ is the mean intensity of reflections with unique indices h . ^b R -factor = $\sum ||F_o| - |F_c|| / \sum |F_o|$.

by taking the absorbance at 280 nm and multiplying by the extinction coefficient of chicken TIM of 1.21 mg/o.d.). A 0.5 M solution of phosphoglycolohydroxamate (PGH) in water was prepared by mixing 2 mg of PGH in 25 μL of MilliQ H_2O . For a 15 mM concentration of PGH in the crystallization drop, 115.2 μL of protein was mixed with 1.8 μL of PGH solution. The S96P-PGH complex was crystallized by vapor diffusion in hanging drops in the presence of saturating amounts of PGH by precipitation with a relatively wide range of poly(ethylene glycol). The standard mother liquor of these crystals contained 100 mM Tris buffer, pH 8.5, containing 12.5–21% (w/v) PEG (8 K), 200 mM lithium sulfate, and 1 mM sodium azide. The crystals of S96P-PGH were nearly isomorphous with the wild-type TIM-PGH crystals. They grew as elongated prisms at room temperature within a week. The crystals were mounted in quartz capillary tubes for data collection. The mutant enzyme crystals have the symmetry of the $P2_12_12_1$ space group, with one dimer per asymmetric unit, and cell dimensions of $a = 136.5 \text{ \AA}$, $b = 74.3 \text{ \AA}$, and $c = 53.5 \text{ \AA}$.

Data Collection and Processing. High-resolution data of the mutant S96P-PGH complex were collected on the San Diego MarkII Multiwire detector system with two multiwire proportional chambers (25–27), at the University of California, San Diego. The S96P crystals diffracted to better than 1.94 \AA resolution. To ensure completeness, we collected the whole data set from nine orientations of the crystal, based on the data collection strategy of Hamlin (28), and containing a total of 33 134 unique reflections out of 70 747 observed reflections. The data were processed using the UCSD data reduction package, giving a completeness of 81% (Table 1).

Molecular Replacement. Because the c -axis of the unit cell for the S96P crystal is about 3.5 \AA shorter than that of the wild-type crystal, one round of R -factor minimization was performed using the molecular replacement program package MERLOT (version 2.3; ref 29) and data in the resolution range 8.0–4.0 \AA ($I/\sigma(I) > 4$). The starting model for the molecular replacement solution was the refined 1.8 \AA

resolution structure of the wild-type triosephosphate isomerase complexed with phosphoglycolohydroxamate (1TPH; ref 8). Five cycles of *R*-factor refinement gave an *R*-factor of 36.9% for the data.

Refinement and Model Building. The refinement of the mutant structure was done using the restrained least-squares method implemented in the program PROLSQ (30, 31) and the simulated annealing and other methods implemented in the program Xplor (32). Interleaved between refinement sessions was model rebuilding on an Evans & Sutherland PS330 graphics system, using the modeling program FRODO (33, 34). Results are given in Table 1.

In the first round of refinement of the S96P mutant structure with PROLSQ, the data were truncated and only reflections between 10.0 and 2.5 Å resolution were used. To minimize the problem of phase bias from the initial model, we deleted coordinates for several residues thought to be involved in enzyme catalysis from the model. These residues were the flexible loop residues (166–176) and the side chains of several active-site residues, including N11, K13, H95, S96, E165, and the PGH molecule. Coordinates for all water molecules in the wild-type TIM–PGH model were also eliminated. After several rounds of PROLSQ the *R*-factor dropped from 37.9% to 27.3%.

Refinement was continued using the program Xplor, using a data set from 10 to 1.94 Å resolution. One round of simulated annealing with a starting temperature of 4000 °C was carried out, resulting in an *R*-factor of 27.4%. Positional refinement, followed by group and individual *B*-factor refinement, reduced the *R*-factor to 24.2%.

A difference Fourier electron density map with coefficients $2F_o - F_c$ was calculated with the truncated model. The first map showed clear density over the whole dimer, including the side chains of E165, H95, P96, N11, and K13, as well as the flexible loop in the closed form. The model was modified by building the 11-residue loop into density, by adding side chains to the active-site residues, and by building in the PGH molecule. Another round of refinement using Xplor resulted in an *R*-factor of 23.9%.

A final round of refinement involved picking of water molecules. Potential candidates were picked from a difference Fourier electron density map with coefficients $F_o - F_c$, based on contour levels at the 3σ level and higher and having reasonable hydrogen-bonding partners. Candidates with *B*-factors of 55 Å² or higher were ultimately eliminated. A total of 281 water molecules were added to the structure. This final model of the S96P–PGH complex was refined against all data from 6 to 1.94 Å resolution giving a final *R*-factor of 20.2% using all observed reflections (Table 1).

FTIR Spectroscopy. Infrared absorbance spectra were recorded using an FTS-40 instrument (Digilab, Cambridge, MA) equipped with a temperature-controlled micro Circle cell (Spectra-Tech, Stamford, CT). The sample compartment was purged with dry nitrogen for several hours after installation of the Circle cell and before injection of the protein samples, to minimize water vapor absorbances in the spectra. The spectral resolution is 2 cm⁻¹, and the number of scans accumulated per Fourier transform was 1024. The time required for data acquisition was 20 min. The samples were cooled to 8 °C. The protein samples were first concentrated to 200–300 mg/mL and then exchanged into deuterated buffer that had been prepared by lyophilizing 100

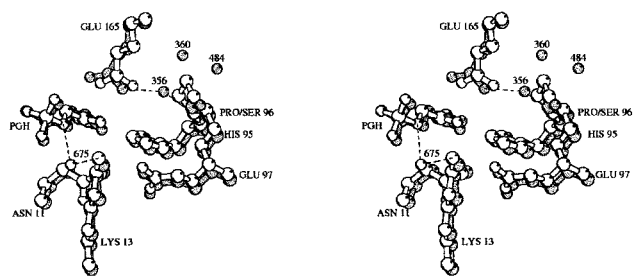


FIGURE 3: Superposition of the active sites of the mutant S96P isomerase (in white) upon the wild-type isomerase (in black). The active-site water molecules are numbered. The figure was prepared using MOLSCRIPT (43).

mM Tris-HCl buffer, pH 7.6, containing EDTA (1 mM), and then dissolving this residue in D₂O. The buffer had a pD of approximately 8. The volume of sample required to fill the Circle cell and connecting tubing was approximately 60 mL. The Circle cell was exhaustively washed with buffer between each filling with sample, cleaned daily with a dilute solution of low-foaming detergent, and fully washed extensively with H₂O. Protein (70 μL, 10 mM final concentration of active sites) was mixed with substrate (4 mL, 14 mM final concentration) immediately before the sample was injected into the Circle cell.

To obtain spectra of enzyme-bound species, we made appropriate subtractions of the spectra of free substrate and of free enzyme. These subtractions have a negligible effect on the region of the spectrum from 1700 to 1760 cm⁻¹, but reduce the absorbance below 1700 cm⁻¹.

RESULTS

Structure of the S96P Mutant of Chicken TIM. The S96P single mutant has an overall structure that is essentially unchanged from that of the wild-type chicken enzyme (Figure 3). The atomic coordinates of the mutant superimpose on those of the wild-type isomerase with root-mean-square deviations in the range 0.2–0.3 Å. On the basis of this comparison and other structures at similar resolution, we estimate that we can detect changes in interatomic distances of greater than 0.2–0.25 Å reliably. When the mutant structure is compared with that of the wild-type, most of the active-site residues show conformations similar to that of the wild-type enzyme structure. Thus, to a first approximation, the effects of the mutation are limited to local changes in side chain positions. Those changes that have occurred are clustered in the active-site region, with the only dramatic conformational changes observed around position 96 itself. Thus, K13, H95, E97, and the flexible loop (residues 166–176) are positioned identically as those in the wild-type enzyme. The substitution of a proline residue at position 96 has no effect on the backbone conformation because the φ angle of S96 in the wild-type structure is already optimal for a proline. In addition, the proline residue is positioned in such a way that the χ_1 torsion angle (the angle around the C_a–C_b bond), is the same as that for serine in the wild-type structure. In the S96P mutant, the proline side chain occupies more space than does the serine side chain in the wild-type, with the result that the tightly bound water molecule that is observed near residue S96 in wild-type TIM is gone. At the same time, since a proline side chain is shorter than a serine side chain, the side chain of

E165 has moved toward the position of P96, and away from the putative position of the substrate. This change in position of E165 is significant. In the wild-type enzyme, the side chain torsion angles for the glutamic acid are $\chi_1 = -35^\circ$, $\chi_2 = -156^\circ$, and $\chi_3 = 101^\circ$, and the carboxylate group is positioned to use its more basic syn orbital (35) for proton transfer with essentially no motion. In the mutant enzyme, the side chain torsion angles for glutamic acid are $\chi_1 = -68^\circ$, $\chi_2 = -174^\circ$, and $\chi_3 = 151^\circ$, and the carboxylate group is positioned in such a way that some rotations would be required for optimal positioning. This change in orientation may be the result of the extra space available to the side chain in the mutant, to changes in interactions due to the loss of the serine side chain, or to changes in the local electrostatic environment in this region of the active site caused by the substitution of a hydrophilic residue by a hydrophobic residue. All of the above could be secondary effects of the loss of the two water molecules normally next to the E165 side chain in the wild-type enzyme. The net effect of this movement is a significant increase in the distance between the closest carboxylate oxygen of E165 and the substrate analogue, PGH, which is the most likely explanation for the 20-fold drop in activity observed in the S96P single mutant.

Conformation and Position of the Intermediate Analogue. In the structure of the wild-type TIM-PGH complex (8), PGH makes a number of interactions with the enzyme that are believed to be representative of those made by substrate. Thus, the NE2 of H95 is positioned midway between the two oxygen atoms of the hydroxamate group, K13 interacts with the phosphate group, and one of the carboxylate oxygens of E165 is positioned 3.4 and 2.6 Å away from the carbon and nitrogen atoms of the hydroxamate, respectively. The intermediate analogue is bound in an extended, planar conformation that would facilitate proton transfer and disfavor the elimination side reactions if substrate bound analogously (7).

These interactions are preserved in the S96P mutant structure, but there are some changes in the relative positions of the groups involved. The PGH molecule has not changed position relative to that in the wild-type structure, but the side chain of E165 has. The carboxylate oxygen atom of this residue is now 3.6 and 2.9 Å away from the carbon and nitrogen atoms of PGH, respectively. The net effect of this movement is to position this oxygen of the carboxylate group as much as 0.4 Å away from its position in the wild-type enzyme. The conformation of the intermediate analogue is unchanged with respect to that seen in the wild-type structure. Thus, the position of the intermediate analogue does not “track” the movement of E165, resulting in a greater distance between PGH and the catalytic base. If this change in relative positioning occurs with the substrate, the base would no longer be positioned optimally for proton abstraction and delivery during the course of the reaction.

Changes in Positions of Bound Water Molecules. Several water molecules are trapped in the active site when PGH binds to wild-type TIM and the loop closes (8). In the wild-type TIM-PGH complex, H₂O-356 accepts a hydrogen bond from the backbone amide of S96 (2.9 Å) and donates hydrogen bonds to H₂O-360 and one of the carboxylate oxygens of E165 (2.7 Å). H₂O-360 donates a hydrogen bond to the backbone carbonyl oxygen of E165 (2.6 Å) and is

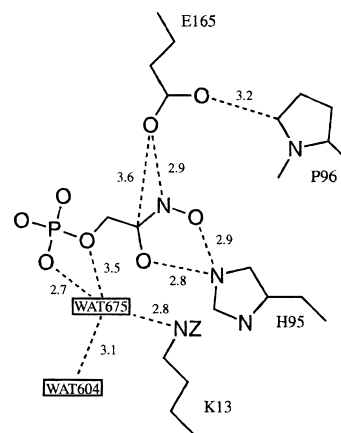


FIGURE 4: Schematic diagram of the hydrogen-bonding pattern in the active site of the S96P mutant enzyme. Only distances less than 3.6 Å are given.

hydrogen-bonded to H₂O-484, which is near the surface of the protein. Thus, the catalytic base is connected to the external solvent by a network of bound water molecules stabilized in part by Ser 96. It is this water structure in the active site that is affected mostly by the substitution of a proline for serine (Figures 3, 4). Not only is the water molecule near the position of residue 96 (H₂O-356 in the wild-type isomerase) no longer present, but also the two other water molecules (waters 360 and 484 in the wild-type isomerase) are no longer present. Presumably, the loss of the backbone NH and side chain hydroxyl group of residue-96, combined with the increased bulk of the proline side chain, leaves no position that can stabilize bound water near the catalytic base. Consequently, the region in which the three waters are observed in the active site of the wild-type isomerase structure seems to be vacant of any water molecules in the structure of the mutant. This observation could result from an actual absence of waters in this region, or the presence of disordered water molecules that do not appear in the electron density. If we assume the former, the environment around the carboxylate in the mutant is less polar and less protic than in the wild-type TIM. We think that the former is more likely because the sites that would be occupied by disordered waters are rather nonpolar and are too small to accommodate a water molecule without rearrangement of the surrounding groups.

The interaction of K13 and the phosphate group of PGH is between the Nz of K13 to the bridging oxygen of the phosphate group in the wild-type enzyme at a distance of 3.4 Å. This interaction is maintained in the S96P mutant enzyme (3.2 Å). However, a new interaction has been introduced by the presence of a water molecule not found in the wild-type enzyme (H₂O-675), within hydrogen-bonding distance of the bridging phosphate group of PGH and the Nz of K13 (3.5 and 2.8 Å, respectively). This water molecule is rigidly fixed in place by two further interactions with one of the oxygens of the phosphate group (2.7 Å) and another water molecule (H₂O-604 at 3.1 Å). The net result is a change in the hydrogen-bonding pattern in this region, but with no obvious movement of either the lysine side chain or the PGH molecule. It is not clear whether such a change in hydrogen-bonding pattern would have any effect on the activity of the enzyme.

Table 2: Infrared Data and Kinetic Constants for the S96P Mutant Isomerase Compared to Those of Wild-type Isomerase

	S96P TIM	wild-type TIM
Carbonyl Stretching Frequencies (in cm^{-1}) ^a		
DHAP (1730 cm^{-1} in soln)	1730	1715, 1730
GAP (1733 cm^{-1} in soln)	1712	NO ^b
Kinetic Constants ^c		
DHAP		
K_m (mM)	0.14 (0.24)	0.38 (0.65)
k_{cat} (s^{-1})	11	600
k_{cat}/K_m DHAP ($\text{M}^{-1} \text{s}^{-1}$)	8×10^4	1.6×10^6
n -fold change relative to wild-type (n)	5×10^{-2}	
GAP		
K_m (mM)	0.0033 (0.087)	0.016 (0.42)
k_{cat} (s^{-1})	64	8300
k_{cat}/K_m GAP ($\text{M}^{-1} \text{s}^{-1}$)	2.2×10^7	5.2×10^8
n -fold change relative to wild-type (n)	4.3×10^{-2}	
PGH		
K_i (mM)	0.5	7.1
arsenate		
K_i (mM)	0.7	11

^a The values for the wild-type isomerase were reported previously (9). ^b NO, not observed. ^c See ref 41. The number in parentheses is the value calculated from the total concentration of DHAP or GAP. Since the actual substrate is the free (unhydrated) form of the substrate, this value has been corrected for the fraction of the substrate that is in the free form.

If we assume that the structurally observed water molecules in the active site are the only waters present, the net effect of the change in water structure within the active site would be to change the type of interaction between the lysine ammonium group of K13, and, more importantly, to change the electrostatic environment around the catalytic base (E165). If we assume that the absence of waters observed is due to disordered waters present, then a connection to the solvent is still possible, as in the wild-type, however, not in the same way. It is not possible to predict how the presence of disordered waters would affect the activity of the enzyme, but for the reasons given above, we do not favor an interpretation in which such waters are present in the equilibrium structure. Their transient presence at any given instant, on the other hand, cannot be ruled out.

Substrate Carbonyl Polarization. Carbonyl group polarization is quantified experimentally as a perturbation of the carbonyl stretching frequency in the ground state of the enzyme-bound substrate. Such ground-state interactions must be maintained in the transition state for TIM-catalyzed proton transfer if they are to contribute to the catalysis of this reaction. The infrared absorbance of the carbonyl group of DHAP, which appears at 1730 cm^{-1} in solution, is shifted to lower wavenumbers (1715 cm^{-1}) when DHAP is bound at the active site of wild-type TIM (Table 2; Figure 5) (9). This shift has been interpreted in terms of the polarization of the carbonyl group of the substrate by interaction with H95. In fact, mutation of this histidine to asparagine or glutamine results in substrate carbonyl stretching frequencies between 1732 and 1742 cm^{-1} , interpreted as complete loss of substrate polarization by the enzyme (10). A stretching frequency due to the carbonyl group of GAP is not observed in the wild-type enzyme, either due to a shift of this frequency below 1700 cm^{-1} , where it is obscured by the protein, or due to an equilibrium that favors predominantly DHAP bound to the enzyme.

The carbonyl stretching frequencies observed when substrate is bound to the S96P mutant are at 1730 and 1712

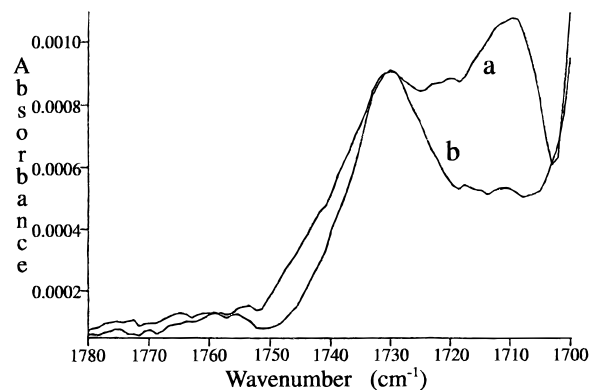


FIGURE 5: Fourier transform infrared spectrum of substrates bound to the single mutant S96P chicken triosephosphate isomerase. The spectrum of unlabeled substrates bound to the isomerase (a) shows bands at 1730 and 1712 cm^{-1} . Labeling the substrates with ^{13}C in the 1-position (b) shows the loss of the GAP carbonyl stretch, which can therefore be assigned to the 1712 cm^{-1} band. Labeling of the substrates with ^{13}C at all positions (not shown) causes the loss of both bands. Thus, the band at 1730 cm^{-1} can be assigned to DHAP.

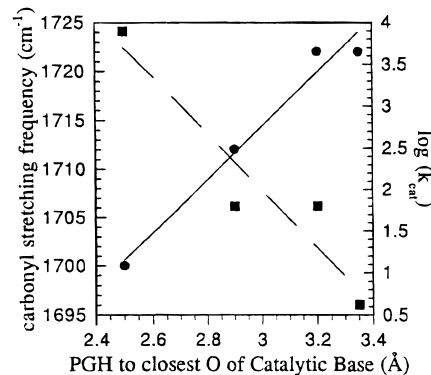


FIGURE 6: Graph of the carbonyl stretching frequency of GAP versus the distance between the nitrogen atom of PGH (mimic of the carbonyl carbon atom of GAP) and the closest oxygen of E(D)165 (solid line, circles) and $\log k_{\text{cat}}$ (dashed line, squares). Distances are the following: wt, 2.5 Å; S96P, 2.9 Å; E165D, 3.2 Å; and E165D/S96P, 3.35 Å.

cm^{-1} , due to DHAP and GAP, respectively. The identities of the observed absorbance bands were determined by selective ^{13}C labeling. In each case, the starting labeled substrate was allowed to equilibrate on the enzyme, so the amount of observed carbonyl for each of DHAP and GAP is related to the internal equilibrium constant for bound GAP versus DHAP. The use of 1- ^{13}C -GAP and U- ^{13}C -DHAP allows unambiguous assignment of both GAP and DHAP carbonyls.

The above result would indicate that the carbonyl of DHAP is less polarized in the S96P mutant than in the wild-type enzyme. It is possible that the presence of the carboxylate oxygen from E165 participates in the carbonyl polarization, pulling on the $\text{C}\delta^+$, while H95 pulls on the $\text{O}\delta^-$. In this case, the distance from the carbon of the substrate to the nearest oxygen of E165 would correlate with the carbonyl stretching frequency. For the set of mutants of E165, S96, and the wild-type, in which the substrate does not seem to be distorted (on the basis of the conformation of PGH bound), this seems to be the case (Figure 6). Furthermore, at least for GAP, polarization correlates inversely with k_{cat} . We recognize that these correlations are based only on a few data points, so it is possible that they may be fortuitous,

or reflect a more subtle feature of the catalytic mechanism. A more general question arises as to whether the catalysis of proton transfer arises primarily from the ground state polarization of the carbonyl group, an effect that tends to bring the structure of the substrate closer to that of the transition state, or from the stabilizing hydrogen bond to the enolate oxygen that develops in the transition state for proton transfer. It seems likely that both effects contribute to the efficient catalysis of proton transfer by TIM, but the relative magnitudes of these contributions are not known.

DISCUSSION

Structural Basis for the Catalytic Lesion in the S96P Mutant TIM. The reaction catalyzed by triosephosphate isomerase is superficially simple, yet many different factors can affect the rate (for an excellent review, see the recent paper by Richard (44)). Thus, mutation of a catalytic residue can alter the reaction rate in a number of ways, many of which are not mutually exclusive. Residue 96 in the active site of triosephosphate isomerase does not seem to be involved directly in the catalytic cycle of the wild-type enzyme. It is too far away from the substrate (as determined by the distance between the serine γ -oxygen atom and the nearest PGH atom in the enzyme PGH complex, 6.9 Å) to be involved in proton shuttling, either from carbon to carbon or from oxygen to oxygen. Therefore it is surprising that the mutation of this residue has any effect at all on the activity of the enzyme. It is even more surprising that, although the mutation of this residue to a proline partially rescues the loss of activity accompanying mutation of the catalytic base, E165, to aspartate, and H95, the proton shuttle, to asparagine, the single S96P mutant is 20-fold reduced in catalytic efficiency.

What is observed crystallographically in the structure of the S96P mutant-PGH complex is a change in the position of the catalytic base, E165, away from the intermediate analogue by as much as 0.4 Å. (We estimate that the smallest distance change that is experimentally significant in structures at this resolution is 0.2–0.25 Å.) If the structure of TIM is indeed evolved to be as efficient as is possible, then the distance between catalytic base and substrate must be ideal for the reaction catalyzed. A change in that distance would result in a less efficient reaction, as is observed for this mutant. Is 0.4 Å sufficient to account for the loss of activity? In part, that question can be answered by examining the structure of the uncomplexed E165D mutant. In this mutant, the carboxylate group of the catalytic base (E165) is pulled back from its position in the wild-type isomerase by 0.7 Å. In addition, the side chain reorients slightly in order to avoid a steric clash with the α -carbon of G209 (11). The activity of the E165D mutant is reduced 300-fold compared to the wild-type isomerase (14). Model building and computational analysis had suggested that the replacement of glutamate by aspartate should increase the distance by 1 Å and that this change in position could account for the observed decrease in activity (36, 37). The S96P mutant has a catalytic efficiency that is reduced 20-fold compared to the wild-type isomerase, and we show here that, on the basis of the structure of the S96P-PGH complex, we expect a 0.4 Å increase in the carboxylate to substrate distance.

Indeed, the change in distance that affects substrate carbonyl polarization is linearly related to $\log k_{\text{cat}}$ (see Figure

6). However, the explanation for the difference in activity is not that simple since the precise position of the carboxylate group is also important. This position depends not only on interactions with Ser 96, but also on the presence around E165 of several bound water molecules. It is possible that the effect of the water is to push or lock the side chain of E165 down on the substrate in the correct orientation. Unfortunately, there is no straightforward correlation between orientation and rate, although some have been proposed in model systems.

Hydrophobic Environment of the Active Site. The absence of three bound water molecules in the active site of the structure of the S96P mutant enzyme structure suggests several other reasons, not mutually exclusive, for the lower activity of this mutant over that of the wild-type. The presence of well-defined water molecules in the active site of the wild-type-PGH complex has been interpreted in terms of the pK_a balance between the catalytic base, E165, and the substrates, DHAP or GAP (8). This balance is required so that the base can act as an efficient proton-transfer agent from one carbon atom of the substrate to another. Because the active site of the uncomplexed enzyme is accessible to solvent (flexible loop open), we assume that the pK_a of the carboxylate of E165 is close to its normal value and that substrate binding and concomitant loop closure leads to an increase of this pK_a due to steric desolvation of the side chain. In aqueous solution, the pK_a of a proton alpha to a carbonyl is in the range of 20, whereas that for a carboxylate side chain is in the range of 5. Experiments with acetic acid as a model compound have shown that the pK_a of the acid changes dramatically with the hydrophobicity of the environment, from a pK_a of 4–5 in aqueous solution, to 9.6 in methanol, to 12.9 in DMSO (38), and to over 50 in vacuo (39). A more dramatic example comes from a study in 82% dioxane/18% water, in which acetic acid has a pK_a of 10.14 (40). Such a change in environment has been used to partly explain the effect of the S96P mutation on the E165D mutant (1). In the structure of the E165D,S96P double mutant complexed with PGH, the altered location of water molecules in the active site may result in changes in the pK_a 's of both the catalytic base and the substrate. A comparison between the E165D single mutant structure and the E165D, S96P double mutant structure, both with PGH bound, shows that the loss of two waters near the base, expected to increase the pK_a of the base, and a gain of a water molecule underneath the substrate, expected to decrease the pK_a of the substrate, would improve the catalytic activity of the double mutant. By achieving a better match of the pK_a 's of the catalytic base and the substrate, the E165D,S96P mutant requires less energy to catalyze the elementary steps of the reaction (4).

The S96P mutant would seem to have provided a considerably more hydrophobic environment in the active site than the wild-type enzyme. It is expected that the pK_a of the catalytic base is increased in such an environment. At the same time, the single water molecule observed near the phosphate group of PGH could have the opposite effect on a substrate bound in this same position, that is, to lower the pK_a of the substrate protons. These changes in pK_a could, in the E165D,S96P double mutant, partially offset the effect of the larger distance and different orientation of the carboxylate of E165D. The infrared spectrum of the substrate

bound to the mutant enzyme would seem to put a limit on the change in pK_a for the side chain of the glutamate residue, since no absorbance is observed attributable to residues of the protein.

The presence of some water molecules in the wild-type active site has the effect of mediating the pK_a of E165 from that in a totally hydrophobic environment. However, the absence of water molecules, that is, a more hydrophobic environment, should have the effect of binding the substrate or one of the intermediates on the reaction pathway, particularly if they are charged, more tightly in the mutant than in the wild-type enzyme, where the charge–charge interactions of active-site residues and putative charged reaction intermediates can be mediated by the presence of water. This idea is supported by the fact that charged inhibitors, arsenate and PGH, are bound more tightly to the S96P mutant enzyme than to the wild-type enzyme (Table 2). We rule out the trivial explanation that the mutation stabilizes nonproductive phosphate binding because all structures show the same position for the phosphate group of PGH, which in turn has previously been shown to be identical to the position of the phosphate group for the substrate, DHAP (37). It would seem, therefore, that the hydrophobicity of the active site can have two effects with opposing consequences on the activity of the enzyme: one is to alter the basicities of the interacting species to match their pK_a 's more closely and thereby improve the activity, and the other is to affect the binding affinity of the reactive species for the active site, with potentially damaging effects on the activity (substrates and/or intermediates bound too tightly for efficient catalysis).

Polarization of the Substrate Carbonyls in the Mutant Isomerases. Comparison of the FTIR spectra of substrates bound to the S96P mutant TIM with that of the wild-type enzyme (9) shows that the substrate carbonyl groups are not as polarized when bound to the mutant enzyme as they are on the wild-type enzyme. The carbonyl stretching frequency for unbound DHAP is found at 1730–1733 cm^{-1} . For the S96P mutant the carbonyl stretching frequency is found at 1730 cm^{-1} . In contrast, the DHAP carbonyl stretching frequencies for the wild-type enzyme are found at 1710 and 1720 cm^{-1} (both of these IR bands have been attributed to DHAP; the carbonyl of GAP is not observed in IR spectra of the wild-type isomerase–substrate complex). Finally, the DHAP carbonyl stretching frequency for the mutant H95Q isomerase, which does not at all polarize the carbonyls of the substrate, is found at 1734 cm^{-1} (10). This comparison indicates that the S96P mutant polarizes the substrate slightly, although nowhere near to the extent of the wild-type. Since the PGH intermediate analogue is bound the same distance from H95 in both the mutant (3.0 and 2.8 Å from H95NE2 to PGH oxygens) and the wild-type structures, it can be inferred that the distances of the substrate carbonyl groups to H95 are not the only factors contributing to substrate carbonyl polarization. The proximity of the catalytic base to the substrate, the positive charge on K13, and ultimately the electrostatic environment near the substrate probably all contribute to the polarization of the substrate DHAP carbonyl.

A carbonyl stretching frequency due to GAP bound to the S96P mutant at 1712 cm^{-1} is a surprise. The carbonyl stretching frequency of unbound GAP is observed at 1733

cm^{-1} . With the wild-type enzyme, a carbonyl stretching frequency attributable to GAP is not observed. Two interpretations have been offered for this latter observation. One is the possibility that the stretching frequency is shifted to below 1700 cm^{-1} , where it is no longer visible due to interference from the protein. The other is that the internal equilibrium on the enzyme favors DHAP over GAP, rendering the contribution of this stretching frequency negligible. For the wild-type enzyme, the former is the generally accepted explanation (9). If this interpretation holds for the mutant S96P, then the observation of an infrared band at 1712 cm^{-1} indicates that the carbonyl of GAP is not as polarized on the mutant as on the wild-type. It is very interesting that the degree to which the carbonyl of GAP is polarized correlates with the distance from the oxygen of E165 and correlates inversely with $\log k_{\text{cat}}$. This remarkable correlation is consistent with kinetic measurements of the individual rate constants for GAP (41).

The Contribution of Water to the Catalytic Activity of TIM. The conformation of the carboxylate group of E165 may very well be controlled by the presence of water molecules in the active site, at least those seen crystallographically. In the wild-type enzyme, one of the carboxylate oxygens of the catalytic base, E165, is hydrogen-bonded to a tightly bound water molecule (H₂O-356), that is in turn hydrogen-bonded to the amide N of S96. This water is also hydrogen-bonded to another water molecule (H₂O-360) that is itself hydrogen-bonded to a third water molecule (H₂O-484). This extensive hydrogen-bonding network provides a bridge between the catalytic base and the external solvent. We hypothesize that this hydrogen-bond network serves two functions: it helps to maintain the position of the carboxylate group relative to the substrate, and it affects the basicity of the carboxylate group. The mutation of S96 to proline has a profound effect on this hydrogen-bond network. None of these water molecules are seen in the electron density maps of the mutant S96P–PGH complex structure, indicating that this hydrogen-bonding network is no longer present, at least with discretely ordered solvent.

There are a number of possible consequences of this change in water structure in the active site. One of these has already been discussed and involves the influence of waters on the hydrophobicity of the active site. The other involves the positioning of the active-site base and substrate for optimal reaction geometry. Changes in the electrostatic environment of the active site would have the consequence of altering the basicity of the catalytic base, in this case increasing the basicity and producing a potentially better catalyst. Movement of the base away from the substrate would have an opposite effect. The balance between these two presumably leads to the observed modest decrease ($20\times$) in k_{cat}/K_m when Ser 96 is changed to Pro in wild-type TIM.

SUMMARY

Our crystallographic and spectroscopic studies of the S96P mutant of cTIM suggest that the loss of activity due to this mutation cannot be explained by a single cause, but instead results from a number of partially compensating factors, including a significant change in the structure of bound solvent in the active site. Free-energy calculations suggest

that this complexity is true in a number of mutant enzymes (42).

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