The Structural Basis for Pseudoreversion of the E165D Lesion by the Secondary S96P Mutation in Triosephosphate Isomerase Depends on the Positions of Active Site Water Molecules[†,‡]

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ABSTRACT: The structural basis for the improvement in catalytic efficiency of the mutant E165D chicken triosephosphate isomerase by the secondary mutation, S96P, has been analyzed using a combination of X-ray crystallography and Fourier transform infrared spectroscopy. All X-ray structures were of the complex of phosphoglycolohydroxamate (PGH), an intermediate analog, with the isomerase, and each was solved to a resolution of 1.9 Å. Comparison of the structure of the double mutant, E165D·S96P, with that of the single mutant, E165D, as well as with the wild-type isomerase shows only insignificant differences in the positions of the side chains in all of the mutants when compared with the wild-type isomerase, except that in both the E165D and E165D·S96P mutants, the aspartate side chain was approximately 0.7 Å further away from the substrate analog than the glutamate side chain. Significant differences were observed in the crystal structure of the E165D·S96P double mutant in the positions of ordered water molecules bound at the active site. The loss of two water molecules located near the side chain at position 165 was observed in isomerases containing the S96P mutation. The resulting increase in hydrophobicity of the pocket probably causes an increase in the pK_a of the catalytic base, D165, thereby improving its basicity. A new ordered water molecule was observed underneath the bound PGH in the E165D·S96P structure, which likely decreases the pK_a's of the substrate protons, thereby increasing their acidity. An enzyme derived carbonyl stretch at 1746 cm^{-1} that is only observed in the IR spectrum of the E165D·S96P double mutant isomerase with bound substrates has been assigned to a stable ground state protonated D165−enediol(ate) intermediate complex. Thus, the gain in activity resulting from the S96P second site change probably results from a combination of improving the basicity of the enzyme, improving the acidity of the substrate protons, and stabilization of a reaction intermediate. All three of these effects seem to be caused by changes in bound water molecules.

Triosephosphate isomerase (TIM) catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP). The reaction rate is limited by the rate of diffusion of substrate onto and off the isomerase surface (Blacklow et al., 1988), and thus TIM is said to have evolved to catalytic perfection (Albery & Knowles, 1976; Knowles & Albery, 1977). Several experimental techniques have been applied to TIM in order to determine its catalytic mechanism and the way in which the structure of the enzyme supports the mechanism. We utilized two such techniques in the work presented here, both of which have been applied previously to wild-type TIM as well as several mutants. X-ray crystallography of the complex of TIM with phosphoglycolohydroxamate (PGH) is thought to give the best approximation of the structure of the enzyme in its catalytically competent form (Davenport et al., 1991; Zhang et al., 1994). Fourier transform infrared spectroscopy (FTIR) has allowed observation of the carbonyl group of the substrates when bound to the enzyme and has proven useful in determining the degree to which the enzyme polarizes the substrate carbonyl groups toward the transition state of the reaction (Belasco & Knowles, 1980; Komives et al., 1991).

The structure of the wild-type isomerase bound to the intermediate analog, PGH, shows the catalytic base, E165, perfectly positioned above the substrate so that the carboxylate is equidistant from the two carbons from which and to which the proton is transferred in the reaction (Zhang et al., 1994). Also involved in catalysis is H95, which serves as a proton shuttle between the two oxygen atoms of the two enediolate intermediates in the reaction and is responsible for polarizing the substrate carbonyl groups toward the transition state of the reaction (Nickbarg et al., 1988; Komives et al., 1991; Bash et al., 1991). Finally, the conserved lysine at position 13 (position 12 in yeast TIM) appears to be responsible for creating a positively charged

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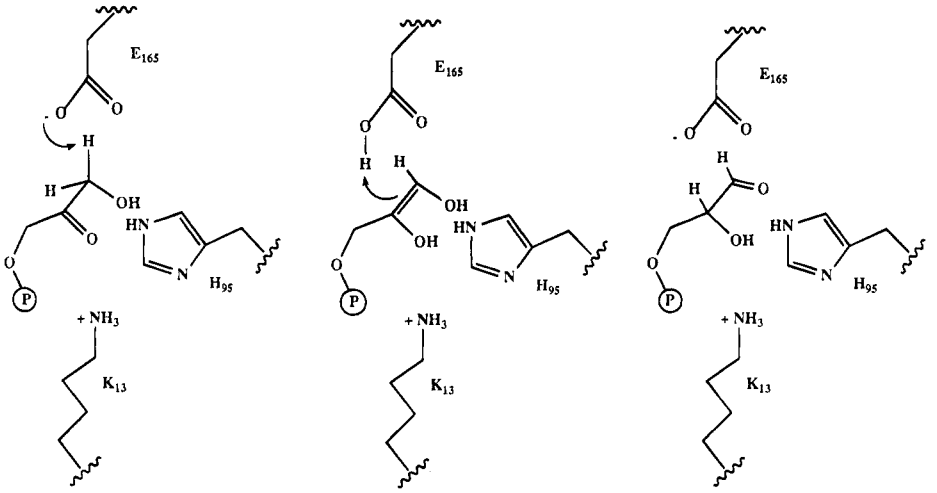

FIGURE 1: Mechanism of the conversion of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate by triosephosphate isomerase.

active site to which the dianionic substrates can bind (Lodi et al., 1994; Joseph-McCarthy et al., 1994a). The two other conserved residues in the TIM active site are E97 and S96, but their roles are uncertain. E97 is within hydrogen-bonding distance of K13 and may serve to hold the lysine side chain in place. Its protonation state is unknown, but based on the close distance between E97 and K13, E97 is most likely ionized. Serine 96, adjacent to the catalytically important H95, is hydrogen-bonded to E165 in the substrate-free form of the isomerase, and this hydrogen-bond is broken when substrate binds and E165 moves by more than 2 Å into position over the two substrate protons that will be isomerized (Lolis & Petsko, 1990). The constellation of active site residues and a proposed catalytic mechanism for the wild-type isomerase are shown in Figure 1.

There is evidence that the pK_a's of both E165 and H95 are perturbed. NMR experiments suggest a pK_a of close to 7 for the catalytic base, E165, perhaps due to solvent exclusion by substrate binding and loop closure (Campbell et al., 1979). The pK_a of H95 has been shown to be less than 4 by NMR (Lodi & Knowles, 1991). This perturbation may arise, in part, from the location of H95 at the N-terminus of a short α-helix (residues 95−102), where it is subject to the positive end of the helix dipole (Lodi & Knowles, 1993).

The single mutant isomerase, E165D, has a catalytic efficiency (k_{cat}/K_m) that is reduced by 300-fold compared to that of the wild-type isomerase (Raines et al., 1986). This mutant isomerase appears to transfer protons to and from substrate and product by the same mechanism as the wild-type isomerase, but the rate-limiting step in the conversion of DHAP to GAP is the deprotonation of DHAP and the rate-limiting step in the conversion of GAP to DHAP is the reprotonation of the enediol to form DHAP. These kinetic experiments were supported by structural studies on the free enzyme (Lolis et al., 1990) as well as on the E165D mutant of yeast TIM complexed to the intermediate analog, PGH (Joseph-McCarthy et al., 1994b), which showed that the overall structure of the enzyme had not changed, but the carboxylate group of D165 was approximately 1 Å further away from the active site. We report here the structure of the E165D chicken muscle triosephosphate isomerase complexed to the intermediate analog, PGH, which is consistent with these previous reports. Although one would expect the yeast and chicken E165D mutant isomerases to have similar structures, the second site pseudoreversion of the E165D mutant by the S96P mutation only improves the catalytic activity of the chicken E165D isomerase, not the yeast E165D isomerase (E. Komives, unpublished data). Thus, in order to understand the structural basis of pseudoreversion and the resulting improvement in catalytic activity, it is necessary to study the structure of the chicken mutant isomerases.

Because TIM appears to have evolved to catalytic perfection, an attempt was made to take the catalytically damaged E165D mutant and by random mutagenesis chart an alternate pathway back to maximum catalytic efficiency. Thus, the E165D chicken muscle triosephosphate isomerase was subjected to random mutagenesis, and second site revertants with improved catalytic activity were isolated. These experiments led to the discovery that the second site alteration of serine 96 to proline (S96P) improved the catalytic potency of the E165D mutant isomerase by 20-fold (Hermes et al., 1990; Blacklow & Knowles, 1990). This double mutant, or pseudoreverted isomerase, was shown to transfer protons to and from substrate and product by the same mechanism as the wild-type isomerase (Blacklow & Knowles, 1990).

We here report a series of X-ray crystallography and FTIR studies aimed at determining the structural basis of the improvement in catalytic activity caused by the pseudoreversion of E165D TIM by the S96P mutation. We present the X-ray structures of the E165D single mutant and the E165D·S96P double mutant isomerase complexed to PGH as well as the carbonyl stretching frequencies of DHAP and GAP bound to the active sites of each mutant isomerase. These data show that the major differences between these mutant isomerases is not in the side chain positions, but rather in the positions of conserved, ordered water molecules at the active site. Water molecules have been implicated in the catalytic mechanisms of many proteins, and it was surprising that the mechanism of TIM did not seem to require any. On the other hand, "wash out" of labels from non-exchangeable sites on the substrate implied that once the proton was removed from the substrate by E165, this proton could exchange with bulk water at a rate that was of the same order of magnitude as k_{cat}. Evidence for the pathway of exchange with the bulk water was obtained from the crystal structures of wild-type chicken and yeast TIMs, which both had two conserved water molecules in a hydrogen-bonding network connecting to E165 (Zhang et al., 1994).

Conserved water molecules in the active sites of enzymes are fairly common occurrences. They are usually ordered and their positions are conserved among members of the same homologous family (Screenivasan & Axelsen, 1992). Buried waters appear to exchange with bulk water although they exchange more slowly than waters on the surface of a protein (Otting et al., 1991). The results presented here imply that subtle changes in the enzyme active site, as are caused by site-directed mutations, can alter the positions of bound water molecules. The alterations in water positions determined from the infrared and structural data allow a consistent interpretation of how the S96P second site change improves the catalytic activity of the E165D mutant isomerase.

EXPERIMENTAL PROCEDURES

Reagents. DL-Glyceraldehyde 3-phosphate diethyl acetal, glucuronolactone, histidine, streptomycin, ampicillin, reduced nicotinamide adenine dinucleotide (NADH), ethylenediaminetetraacetic acid (EDTA) disodium salt, Dowex-50W (H^+ form), and QAE Sephadex A-120 were 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 1/10th the volume of H_2O, so that the resulting solution was 10 times as concentrated. Phosphoglycolohydroxamate was prepared by J. Belasco. Bromohydroxyacetone phosphate (BHAP) was prepared as described by de la Mare et al. (1972). 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 hydrochloride, pH 7.6, and 1 mM EDTA. The dialyzed protein was then treated with a 100-fold molar excess of BHAP for 1 h on ice and Centriconed with three buffer changes to remove the excess 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. (1991).

Protein Production. The gene for each mutant was prepared by mutagenesis of the gene for wild-type TIM from chicken muscle contained in a phagemid vector that was a derivative of pBS+/− and has been described (Blacklow et al., 1988). This phagemid vector allowed the efficient production of single-stranded DNA for mutagenesis. The phagemid contains, on an *Eco*RI to *Pst*I fragment, the *trc* promoter upstream from the complete TIM gene and allowed the production of 50−80 mg of protein/L of cells. The expression vectors were used to transform *Escherichia coli* strain DF502, which is a strep^R, tpi$^-$ strain that was kindly provided by D. Fraenkel and has been previously described (Straus & Gilbert, 1985).

Large amounts of pure protein were prepared by growing the bacterial transformants in a final volume of 10 L of M63 salts (Miller, 1972) containing casamino acids (0.5% w/v), glucuronolactone (0.4% w/v), glycerol (0.1% w/v), $MgSO_4$ (1 mM), thiamine (1 mg/mL), L-histidine (80 mg/L), streptomycin (100 mg/L), and ampicillin (200 mg/L). Cells were harvested after 12−20 h by centrifugation at 3000*g*. The cells were lysed in a continuous flow French pressure cell (Aminco, Urbana, IL), and the lysate was centrifuged at 8500*g* 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 proteins were finally purified on a MonoQ 10/10 column using the same gradient. Purity of the proteins was assessed by silver staining overloaded 15% SDS−PAGE gels (Laemmli, 1970). Concentration of the protein was afforded 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 Blacklow and Knowles (1990), and the k_{cat} and K_m values obtained were identical to those previously reported.

Protein Crystallization. The purified protein was dialyzed into MilliQ H_2O and concentrated to 20 mg/mL (calculated by taking the absorbance at 280 nm and multiplying by the extinction coefficient of chicken TIM of 1.21 mg/OD). 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 1.5 M final concentration of PGH in the crystallization drop, 115.2 μL of protein was mixed with 4.8 μL of PGH solution. Initially, crystallization conditions were screened using vapor diffusion of hanging drops by the method of Jancarik and Kim (1991). Crystals appeared at room temperature after 4−7 days in the well containing 100 mM Tris buffer, pH 8.5, 200 mM lithium sulfate, and PEG 8000. Crystals grew at a variable range of PEG 8000 concentrations, and we routinely set up several wells containing concentrations of PEG 8000 from 12.5% to 21% in 2.5% increments. The crystals were mounted in quartz capillary tubes with mother liquor containing PGH on either side to prevent the crystal from drying out. Several crystals were obtained from this range of PEG concentrations, but the exact concentration of PEG 8000 was not reproducible. The crystals grow as elongated prisms and belong to the space group $P2_12_12_1$, with one dimer per asymmetric unit and cell dimensions of $a = 136.4$, $b = 74.1$, $c = 57.2$ Å.

X-ray Crystallographic Data Collection and Processing. For the single mutant E165D isomerase complex with PGH, a high resolution data set was collected on the San Diego Mark II Multiwire area detector system with two multiwire proportional chambers at the Center for Advanced Biotechnology and Medicine and the University of Medicine and Dentistry of New Jersey (Cork et al., 1973; Xuong et al., 1985a,b). The crystals diffracted beyond 1.92 Å resolution. To assure completeness, the whole data set was collected from 18 orientations of the crystal and contained a total of 41 619 unique reflections out of 137 066 recorded measurements. The data set was processed using the UCSD data reduction package giving an R_{sym} of 6.0% on intensities and a completeness of 93% within the resolution range between 11.6 and 1.92 Å (Table 1).

For the double mutant E165D·S96P isomerase, a high resolution data set was collected on the Siemens X100 area detector at Heidelberg, Germany. The crystal diffracted to 1.9 Å resolution. Among a total of 107 422 measurements,

Table 1: Statistics of Data Collection and Refinement for the E165D and E165D·S96P Mutant Isomerase Structures[a]

	E165D TIM	E165D·S96P TIM
Data Collection		
unit cell parameters (Å)	$a = 136.4$	$a = 136.4$
	$b = 74.1$	$b = 73.9$
	$c = 57.2$	$c = 55.9$
space group	$P2_12_12_1$	$P2_12_12_1$
resolution range (Å)	11.6−1.9	50.0−1.9
no. of total reflections	137 066	107 422
no. of unique reflections	41 619	37 566
R_{sym} (%)	6.0	8.5
data completeness (%)	94	83
Refinement		
resolution range (Å)	6.0−1.9	6.0−2.0
R-factor (%)	18.2	17.4
no. of reflections ($I/\sigma(I) > 1$)	38 793	28 952
no. of protein atoms	3710	3712
no. of inhibitor atoms	20	20
no. of water molecules	253	319
deviation from ideal geometry (rmsd)		
bond distances (Å)	0.016	0.015
angle distances (deg)	1.6	1.2
planar distances (Å)	0.047	0.042

[a] $R_{sym} = \sum(|I(h,j) - I(h)|)/\sum(I(h,j))$, where $I(h,j)$ are symmetry-related intensity observations and $I(h)$ is the mean intensity of reflections with unique indices h. R-factor $= \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|$.

37 566 spots were unique reflections within the resolution range between 50.0 and 1.9 Å, giving a completeness of 83% and R_{sym} of 8.5%. The data were reduced with XDS and SCALE (Kabsch, 1988). The structure of the single mutant S96P isomerase will be presented elsewhere (Zhang et al., in preparation).

Refinement and Model Building. Refinement of the coordinates of the single mutant and double mutant isomerase−PGH complexes was done by restrained least-squares with the program PROLSQ (Hendrickson & Konnert, 1980; Hendrickson, 1985) on a Stardent 2000 workstation. Interleaved between refinement sessions were model rebuilding sessions on an Evans & Sutherland PS330 graphics system using the modeling program FRODO (Jones, 1978; Sack, 1988). The procedure for both mutant structure refinements was exactly the same. Phases were calculated from the wild-type chicken TIM−PGH (Zhang et al., 1994) model coordinates with the PGH, active site residues (N11, K13, H95, S96, and E165), flexible loop residues (166−176), and water molecules deleted. A difference Fourier electron density map with coefficient $(3R_o - 2F_c)$ was calculated using all data from 10.0−2.5 Å resolution. The map showed clear electron density over the whole of the dimer model including well-defined densities for the inhibitor (PGH), the active site residues omitted from the model, and the flexible loop residues in the "closed" position. The majority of the manual modifications to the model consisted of introducing two PGH molecules into the active sites of the two subunits and building in the two 11-residue "lid" loops which close over the active site upon PGH binding (Lolis et al., 1990).

In the second stage, a round of PROLSQ with overall B-factor refinement was performed using the same resolution range of data. Then the higher resolution data up to 1.9 Å were gradually added in every few cycles, and the refinement was continued on the newly-built model. Another $(3F_o - 2F_c)$ electron density map was calculated based on the entire data set. Aspartate 165 in the case of the single mutant, or aspartate 165 and serine 96 in the case of the double mutant, as well as the other active site residues that had been omitted from the starting model, were built in to fit the corresponding electron density. After addition of the higher resolution data, an individual isotropic B-factor for each atom was used in the refinement. Following refinement, another round of manual rebuilding based on difference Fourier maps with coefficients $(3F_o - 2F_c)$ and $(F_o - F_c)$ was performed to check the whole dimeric TIM structure, giving an R-factor of 23.1% for the single mutant and 22.7% for the double mutant, respectively.

The final stage of refinement involved picking water molecules. A difference Fourier map with coefficients $(F_o - F_c)$ was calculated, and the potential water candidates were picked based on peaks at the 3* sigma level and higher with reasonable hydrogen-bonding partners. A checking procedure after refinement eliminated those candidates with B-factors higher than 50.0 Å^2. The final model of each mutant TIM complexed with PGH was refined against all of the merged area detector data between 6.0 and 1.9 Å resolution, using all reflections with $I/\sigma(I) > 1$. The final R-factors of the E165 and E165D·S96P mutant TIM−PGH structures were 18.2% and 17.4%, respectively (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 N_2 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 was 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 mM Tris-HCl buffer, pH 7.6, containing EDTA (1 mM), and then dissolving this residue in D_2O. The buffer had a pD of approximately 8. The volume of sample required to fill the Circle cell and connecting tubing was approximately 60 μL. The Circle cell was exhaustively washed with buffer between each filling with sample and cleansed daily with a dilute solution of low-foaming detergent, and finally washed extensively with H_2O. Protein (70 μL, 10 mN final concentration of active sites) was mixed with substrate (4 μL, 14 mM final concentration) immediately before the sample was injected into the Circle cell.

To obtain spectra of enzyme-bound species, appropriate subtractions of the spectra of free substrate and of free enzyme were made. 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 E165D and E165D·S96P Chicken TIM. Both the E165D single mutant and the E165D·S96P double mutant have an overall structure that is essentially unchanged from that of the wild-type chicken enzyme. The atomic coordinates of all of the mutants superimpose on each other and on the wild-type isomerase with an overall root mean squared deviation of 0.2−0.3 Å. The isomorphism persists in the

active site, where the backbone conformation of even the mutated residues is unchanged. Thus, to a first approximation, the effects of the mutations are expressed solely as local changes in side chain position. Most of the side chains in the active site do not move in the single mutant when E165 is changed to aspartic acid. In particular, K13, S96, and E97 and the flexible loop (residues 166−176) are positioned identically as in the wild-type structure. Histidine 95 tilts slightly from its wild-type position: the motion is in the direction of the carboxylate of D165 and presumably occurs because there is more room. The only other significant change in the single mutant structure relative to the wild-type is in the position of the catalytic base, D165. In the wild-type enzyme, the side chain torsion angles for the glutamic acid are $X_1 = -35°$, $X_2 = -156°$, $X_3 = 101°$, and the carboxylate is positioned to use its *syn* orbital for proton transfer with essentially no motion. In the mutant enzyme, the side chain torsion angles for aspartic acid are $X_1 = -86°$, $X_2 = -178°$, and the carboxylate is tilted such that some rotation would be required for optimal positioning. This change in orientation is necessitated by the close proximity of the carboxylate moiety to the backbone of glycine 209. If the effect of the mutation were simply to move the carboxylate back toward its own backbone by 1 Å, there would be a severe collision with the α-carbon of G209. That region of the protein cannot adjust because it is hydrogen-bonding to the phosphate group of PGH. Hence, the carboxylate of D165 reorients to avoid the steric clash. The net effect of these movements is a small increase in distance between the closest carboxylate oxygen of D165 and the substrate analog, PGH.

The active site of the double mutant is very similar to that of the wild-type and single mutant. The positions of K13, E97, and the flexible loop are unchanged. Histidine 95 has tilted back to its wild-type position, presumably due to steric interaction with the newly introduced proline side chain. Aspartic acid 165 is in a similar position to that in the single mutant structure: $X_1 = -113°$, $X_2 = -178°$. The small change in X_1 causes the carboxylate to be oriented at an angle relative to the PGH that is more like that seen in the wild-type structure. The major change in the protein structure is the introduction of the bulkier proline side chain in the active site pocket. Substitution of a proline 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.

Changes in the Conformation and Position of the Intermediate Analog. In the structure of the wild-type TIM−PGH complex (Zhang et al., 1994), PGH makes a number of interactions with the enzyme that are believed to be representative of those made by substrate. The N^{ϵ} of H95 is positioned midway between the two oxygen atoms of the hydroxamate group, K13 is interacting with the phosphate group, and the carboxylate oxygens of E165 are positioned 3.4 and 2.6 Å away from the carbon and nitrogen atoms of the hydroxamate, respectively. The intermediate analog is bound in an extended, planar conformation that would facilitate proton transfer and disfavor the elimination side reaction if substrate were bound analogously (Davenport et al., 1991).

These interactions are preserved in both the E165D and E165D·S96P structures, but there are some shifts in the relative positions of the groups involved. In both the single and double mutants, the carboxylate oxygens of E165 are 4.1 and 3.2 Å away from the carbon and nitrogen of the hydroxamate, respectively. Although the conformation of the intermediate is unchanged with respect to that seen in the wild-type structure, in both mutant structures, the PGH pivots about its phosphate group by approximately 15° relative to its position in the wild-type structure. This shift in the position of the hydroxamate group brings the carbon and nitrogen atoms closer to the carboxylate oxygens of D165 than they would be if the PGH remained in the same position as in the wild-type isomerase. Thus, the net effect of the intermediate analog's "tracking" the change in the carboxylate position is to offset partially the loss of proximity of the catalytic base. In the E165D single mutant, H95 shifts position with the PGH molecule, but in the E165D·S96P double mutant, the bulky proline side chain pushes the imidazole back toward its original position.

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 (Zhang et al., 1994). In the wild-type TIM−PGH complex, H_2O 356 accepts a hydrogen-bond from the backbone amide of S96 (distance = 2.9 Å) and donates hydrogen-bonds to H_2O 360 and to the Oε2 carboxylate oxygen of E165. H_2O 360 donates a hydrogen-bond to the backbone carbonyl oxygen of E165 (distance = 2.6 Å) and is also hydrogen-bonded to water 484, which is near the surface of the protein. Thus, the catalytic base is connected to the solvent by a network of bound water molecules.

In the E165D single mutant, the water molecules just described are still present, and they make the same interactions with the enzyme and with each other. H_2O 379 (corresponding to H_2O 356 in the wild-type protein) is hydrogen-bonded to the Oδ1 carboxylate oxygen of D165 (distance = 2.8 Å) and to H_2O 390 (corresponding to H_2O 360 in the wild-type protein) and the amide of S96 (distance = 2.7 Å). H_2O 390 is still hydrogen-bonded to the backbone carbonyl oxygen of residue 165 and to H_2O 512 (corresponding to H_2O 484 in the wild-type protein). There has been a slight shift in the positions of all three water molecules to accommodate the altered positions of the catalytic base, H95, and the hydroxamate of PGH, but each is within 0.5 Å of its location in the wild-type enzyme. Figure 2 shows the active site of the E165D mutant isomerase superimposed on that of the wild-type isomerase.

Mutation of serine 96 to proline displaces H_2O's 356/379 and 360/390 completely (Figure 3). These water molecules are missing from their positions near the catalytic base in both the single mutant, S96P isomerase (Zhang et al., in preparation), and in the double mutant E165D·S96P isomerase. The loss of the backbone NH of residue 96 combined with the increased size of the proline side chain leaves no position that can stabilize bound water near the catalytic base. As a result, the environment of the carboxylate in the TIMs possessing the S96P mutation is presumably less polar and certainly less protic than in the wild-type and other TIM mutants.

A new water molecule, number 326, is present in the active site of only the E165D·S96P double mutant, not in either the E165D or the S96P single mutant isomerases. H_2O 326 occupies a unique position underneath the substrate and is within hydrogen-bonding distance of the Nε of H95 (distance = 3.3/3.4 Å), the side chain amide NH of N11 (distance = 2.4/3.1 Å), O1 of PGH (distance = 2.4/2.1 Å), O2 of PGH

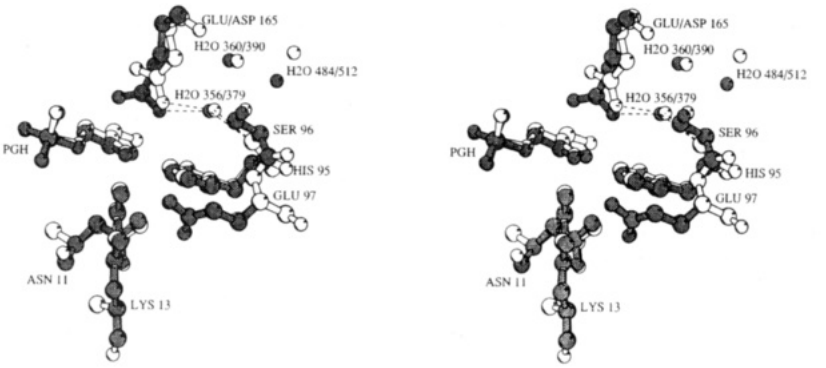

FIGURE 2: Superposition of the active sites of the single mutant E165D isomerase (in white ball-and-stick) upon the wild-type isomerase (in dark gray). The important active site water molecules are numbered, with the first number corresponding to the wild-type isomerase and the second number corresponding to the mutant isomerase. The figure was prepared using MOLSCRIPT (Kraulis, 1991).

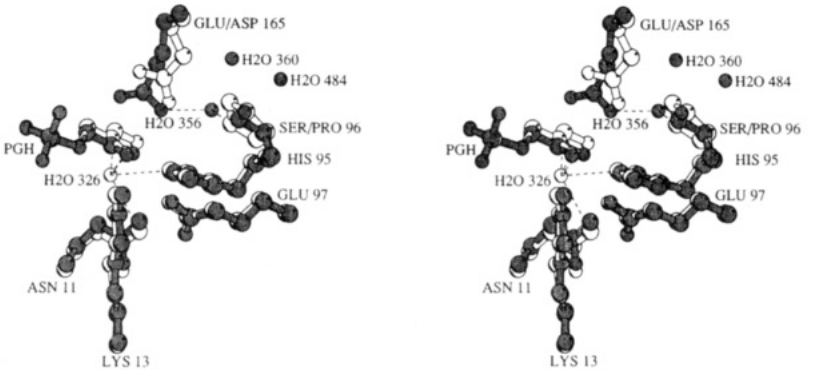

FIGURE 3: Superposition of the active sites of the double mutant E165D·S96P isomerase (in white ball-and-stick) upon the wild-type isomerase (in dark gray). The two water molecules in the wild-type structure, 356 and 360, are missing from the mutant isomerase structure, and a new water molecule, number 326, is present only in the mutant isomerase structure. The figure was prepared using MOLSCRIPT (Kraulis, 1991).

Table 2: Distances (in Å) between Atoms within the Active Sites of the E165D and E165D·S96P Mutant Isomerases Compared to Those of the Wild-Type Isomerase

	E165D TIM		E165D·S96P TIM		wild-type TIM	
N of PGH to Oδ1 of D165[a]	2.9[b]	3.0	2.9	3.1	3.1	3.1
O1 of PGH to Oδ1 of D165	2.4	2.4	2.5	2.6	2.8	2.6
N1 of PGH to Oδ2 of D165	3.2	3.5	3.2	3.2	2.5	2.5
C1 of PGH to Oδ2 of D165	4.1	4.3	4.1	3.8	3.3	3.3
Nϵ of H95 to O1 of PGH	2.9	2.8	2.8	2.9	2.9	2.9
Nϵ of H95 to O2 of PGH	2.8	2.7	2.6	2.6	2.8	2.8
Nζ of lysine 13 to O2 of PGH	2.9	2.7	2.8	3.0	3.3	3.3
water 356/379[c] to N of S96	3.0	3.9	not present		2.8	2.9
water 356/379[c] to Oδ1 of D165	3.0	2.9	not present		2.8	2.7
water 326 to O1 of PGH	not present		2.4	2.1	not present	
water 326 to O2 of PGH	not present		2.7	2.7	not present	
water 326 to C1 of PGH	not present		3.0	2.5	not present	
water 326 to N of PGH	not present		2.9	2.2	not present	
water 326 to Nϵ of H95	not present		3.3	3.4	not present	
water 326 to amide NH of N11	not present		2.4	3.1	not present	

[a] The reported distance is to Oϵ1 of E165 for the wild-type isomerase. [b] The first number corresponds to the distance measured in subunit 1 of the dimeric isomerase, and the second number corresponds to the distance measured in subunit 2. [c] Water 356 is in subunit 1 of the wild-type isomerase. Water 379 is in subunit 1 of the E165D mutant isomerase and is in the same position as water 356 in the wild-type structure.

(distance = 2.7/2.7 Å), N of PGH (distance = 2.9/2.2 Å), and C1 of PGH (distance = 3.0/2.5 Å). It is striking that this water molecule seems to be located squarely under the substrate analog and yet the distances between H_2O 326 and the atoms of PGH are quite short compared to the distances between the atoms of PGH and the carboxylate oxygens of D165 (distances = 3.2/3.2 Å to N of PGH and 4.1/4.3 Å to C1 of PGH). This H_2O molecule has a *B*-factor comparable to those of the PGH atoms and well-positioned protein atoms. The *B*-factor of H_2O 326 is also comparable to that of H_2O 360 in the wild-type isomerase when placed on the same scale. Figure 3 shows the superposition of the active sites of the E165D·S96P double mutant isomerase upon the wild-type isomerase. H_2O 326 can be seen below the substrate analog, PGH bound at the active site. The distances from this water to the active site residues are listed in Table 2.

FTIR Spectroscopy. The FTIR spectra of an equilibrium mixture of DHAP and GAP bound to the E165D single mutant TIM and the E165D·S96P double mutant TIM are shown in Figure 4, panels A and B, respectively. The

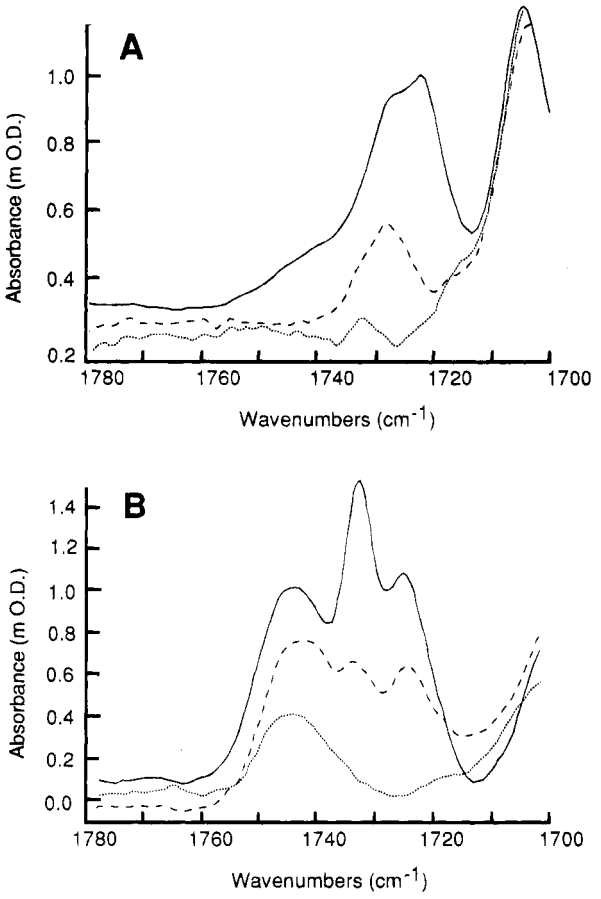

FIGURE 4: (A) Fourier transform infrared spectrum of substrates bound to the single mutant E165D isomerase. The spectrum of unlabeled substrates bound to the isomerase (solid line) shows bands at 1722 and 1728 cm^{-1}. Labeling of the substrates with ^{13}C in the 1-position (dashed line) shows the loss of the GAP carbonyl stretch, which can therefore be assigned to the 1722 cm^{-1} band. Labeling of the substrates with ^{13}C at all positions (dotted line) causes the loss of both bands. Thus, the band at 1728 cm^{-1} can be assigned to DHAP. (B) Fourier transform infrared spectrum of substrates bound to the double mutant E165D·S96P isomerase. The spectrum of unlabeled substrates bound to the isomerase (solid line) shows bands at 1722, 1732, and 1746 cm^{-1}. Labeling of the substrates with ^{13}C in the 1-position (dashed line) shows the loss of the GAP carbonyl stretch, which can therefore be assigned to the 1732 cm^{-1} band. Labeling of the substrates with ^{13}C at all positions (dotted line) causes the loss of the band at 1722 cm^{-1}, which can therefore be assigned to DHAP. The band at 1746 cm^{-1} persists even with completely labeled substrates and can therefore be assigned to an enzymic carbonyl.

identities of the observed absorbance bands were determined by selective ^{13}C labeling, and the spectra observed for the equilibrium mixture of 1-^{13}C substrates bound to the active site of each mutant isomerase as well as the U-^{13}C substrates bound to each isomerase. The starting labeled substrate was allowed to equilibrate on the enzyme surface, so the amount of observed carbonyl for each of DHAP and GAP is related to the internal equilibrium constant for bound GAP versus bound DHAP. Thus, starting with [1-^{13}C]GAP produces both [1-^{13}C]DHAP, for which the carbonyl is still visible, and [1-^{13}C]GAP, for which the carbonyl stretching frequency drops by some 40 cm^{-1} into the region below 1700 cm^{-1}, in which the peptidyl amide carbonyl resonances obscure its observance. Thus, the carbonyl stretch of GAP can be identified as that which disappears when the enzyme is incubated with [1-^{13}C]GAP, and the carbonyl stretch of DHAP can be identified as that which disappears when the enzyme is incubated with [U-^{13}C]DHAP. This method was used previously to determine the identity of the GAP and DHAP carbonyl stretches in the H95Q mutant yeast isomerase (Komives et al., 1991). Resonances that are still observed when the enzyme is incubated with [U-^{13}C]DHAP can be attributed to enzymic carbonyl groups that are perturbed upon substrate binding so that they now absorb in the region below 1700 cm^{-1}.

DISCUSSION

Structural Basis for the Catalytic Lesion in the E165D Mutant TIM. When glutamate 165 is changed to aspartate, the catalytic activity of chicken muscle triosephosphate isomerase is reduced 300-fold. Analysis of the free energy profile of the reaction catalyzed by the E165D mutant shows that proton transfer has become rate-limiting (Figure 5). The mutation was designed to assess the importance of the proximity of the catalytic base to the substrate in the TIM reaction. Model building and computational analysis suggested that the replacement of glutamate at position 165 by aspartate should reduce the distance from the base to the substrate by approximately 1 Å and that this could account for the observed decrease in catalytic activity (Alagona et al., 1986). The high resolution structure of the complex of the E165D mutant TIM complexed with PGH suggests that this hypothesis is essentially correct. Although the model building failed to predict the shift in the position of the PGH that tracks the backward movement of the carboxylate, the shift only partly compensates for the shorter side chain of aspartate relative to glutamate. The distance between the base and the substrate is still 0.7 Å longer than in the wild-type enzyme. A lower resolution study of the complex of the yeast E165D mutant TIM complexed to PGH showed the same effects, suggesting that these displacements are not the result of crystal packing (Joseph-McCarthy et al., 1994b).

Structural Differences between the E165D·S96P Double Mutant and the E165D Single Mutant TIM. Replacement of serine 96 by proline in the E165D mutant TIM results in a partial restoration of catalytic activity by about 20-fold. Comparison of the structure of the E165D·S96P double mutant with those of the E165D single mutant and the wild-type isomerases suggests that the improvement in catalytic activity in the double mutant is *not* due to changes in the structure of the enzyme nor to changes in the position of the substrate. PGH is positioned identically in the single and double mutants. Although H95 and D165 both change position slightly in the E165D·S96P double mutant, these changes are small and do not appear to have an obvious effect on proton transfer rates.

The strikingly unique features of the E165D·S96P double mutant are the loss of the two ordered water molecules near D165 and the gain of a new ordered water molecule underneath the substrate. The fact that ordered water molecules are not observed could indicate either that there are still water molecules there, but they are disordered, or that the lifetime of the water molecules has decreased substantially. Crystallography cannot distinguish between these two possibilities. It is possible to infer, however, that, if an ordered water molecule is no longer observed, the environment has changed enough to disfavor the ordered presence of the water molecule. The loss of two ordered water molecules would therefore be likely to change the

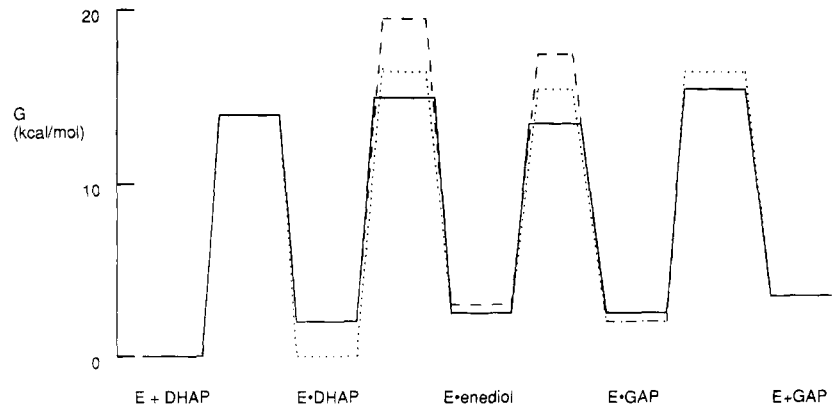

FIGURE 5: Free energy profiles for the wild-type (solid line), single mutant E165D isomerase (dashed line), and double mutant isomerase (dotted line). The generation of these profiles was reported previously (Blacklow & Knowles, 1990).

environment of D165, making it more nonpolar and certainly nonprotic compared to the wild-type and E165D mutant isomerases. The new water molecule (number 326) that is located on the opposite face of the PGH molecule from D165 also probably influences the catalytic activity of the E165D·S96P double mutant isomerase. The distances between H_2O 326 and the atoms of PGH are quite short compared to the distances between the atoms of PGH and the carboxylate oxygens of D165. One would have expected that the presence of this water molecule would have "pushed" the PGH closer to the carboxylate of D165, but the electron density clearly shows the water molecule closely spaced underneath the PGH molecule, and the distance from the PGH to the carboxylate of D165 is the same as that in the single E165D mutant. It is possible that, during catalysis, the substrate is, in fact, "pushed" by this water molecule closer to the catalytic base. Nonetheless, the presence of H_2O 326 is expected to make the environment around the PGH and by inference around the substrate more protic and therefore more polar.

Polarization of the Substrate Carbonyls in the Mutant Isomerases. Comparison of the FTIR spectra of substrates bound to the two mutant TIMs with that of the wild-type enzyme (Belasco & Knowles, 1980) shows that the substrate carbonyl groups are not as polarized when bound to the mutant enzymes as in the wild-type enzyme. Still, both mutants polarize the substrates somewhat in comparison to unbound DHAP, for which the carbonyl stretching frequency is observed at 1733 cm^{-1}. The single E165D mutant and the double E165D·S96P pseudorevertant show similar carbonyl stretching frequencies for both substrates. For the E165D mutant, the DHAP carbonyl stretch is found at 1722 cm^{-1} and the GAP carbonyl stretch at 1728 cm^{-1}, and for the E165D·S96P double mutant, the DHAP carbonyl stretch is found at 1732 cm^{-1} and the GAP carbonyl stretch at 1722 cm^{-1}. These carbonyl stretching frequencies are intermediate between those observed for the wild-type enzyme, 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), and the H95Q mutant isomerase, for which the carbonyl stretching frequencies are found at 1740 cm^{-1}. Since the PGH intermediate analog is bound the same distance from H95 in both the mutant and wild-type structures, it can be inferred that the distance of the substrate carbonyl groups to H95 must not be the only factor contributing to substrate carbonyl polarization. The proximity of the catalytic base to the substrate as well as the positive charge on K13 probably also contribute to polarization of the substrate carbonyl groups. Finally, since both the E165D single mutant and the E165D·S96P double mutant show quite similar carbonyl stretching frequencies for both substrates, the improvement in catalytic efficiency by the second site mutation of serine 96 to proline cannot be explained by an improvement in polarization of the substrate carbonyl groups.

Identity of the Enzymic Carbonyl Absorbing at 1746 cm^{-1}. A prominent carbonyl absorbance was observed in the spectrum of substrates bound to the E165D·S96P mutant isomerase. This absorbance band at 1746 cm^{-1} was identified as an enzymic carbonyl group for which the carbonyl stretching frequency shifts upon substrate binding (Figure 4B). No enzymic carbonyl absorbance bands were observed in the FTIR spectra of either the E165D single mutant (Figure 4A) or the S96P single mutant (data not shown). The only carbonyl group present in a protein that could have a stretching frequency near 1745 cm^{-1} is a protonated carboxyl group in a nonpolar environment. In this special case, carboxylic acids have carbonyl stretching frequencies near 1750 cm^{-1} (Avram & Meteescu, 1972). Since the absorbance was only observed upon substrate binding, it could not be attributed to a permanent chemical modification of the enzyme. The observed band was therefore attributed to a carboxylic acid side chain. A similar enzyme-derived carbonyl stretch at 1748 cm^{-1} was observed in the yeast 1,6-bisphosphate aldolase in the presence of substrate and K^+ ions and was also attributed to an enzymic carboxyl group in a nonpolar environment (Belasco & Knowles, 1983).

Examination of the crystal structure of the E165D·S96P mutant isomerase shows that the only carboxyl side chains within 10 Å of the active site are D165, the catalytic base, and E97, which is within hydrogen-bonding distance of lysine 13. It is possible that the environment of E97 is different in solution than in the crystal, but taking only the crystal data, the fact that a new unique water molecule is present close to E97 in the E165D·S96P double mutant isomerase suggests that, if anything, the environment around this side chain should be more polar rather than less polar.

The most likely possibility is that the enzymic carbonyl that is observed in the infrared spectrum is actually that of protonated D165. This suggestion fits well with the observation that the environment around D165 is less polar in the absence of the two water molecules that are hydrogen-bonded to the D165 in the single mutant enzyme and lends further credence to the suggestion that these water molecules are not just disordered, but are in fact no longer there. Second,

the E165D·S96P mutant binds DHAP much more tightly than the wild-type or the E165D single mutant (Figure 5). Third, exchange−conversion experiments on the E165D·S96P double mutant show a high discrimination for tritium in the GAP to DHAP direction, indicating that reprotonation of the enediol to form DHAP is a slow step in the reaction (Blacklow & Knowles, 1990). Finally, measurements of isotope effects as a function of viscosity for the wild-type enzyme convincingly showed that deprotonation of substrate in the TIM mechanism is coupled to loop closure (Sampson & Knowles, 1992). Thus, the first intermediate on the TIM reaction pathway may be an enediolate and protonated E165 rather than enzyme-bound DHAP. Since infrared spectroscopy has the capability of observing all stable ground state species, it might be possible to observe a stable intermediate in which the substrate proton has already been removed by D165.

Whether the intermediate is the enzyme-bound enediolate or the enzyme-bound enediol is a matter for speculation. Both theoretical and NMR experiments have suggested that the enzyme avoids an enediol intermediate by utilizing a neutral histidine as the proton shuttle between the two oxygens of the two enediolate intermediates (Bash et al., 1991; Lodi & Knowles, 1991). Since an enzymic carbonyl is not observed in the IR spectra of substrates bound to the S96P single mutant, in which E165 is expected to be in a similarly nonpolar environment as is D165 in the E165D·S96P double mutant, one might ask what the differences are between these two mutants. The major difference is that the new water molecule, H_2O 326, is only present in the E165D·S96P double mutant. This water molecule is located only 2.7 Å away from the PGH O2, which is the oxygen that would be ionized in the enediolate formed from deprotonation of DHAP. Thus, this unique water molecule may protonate the enediolate intermediate, forming the more stable enediol intermediate. Although the free energy profile for the E165D·S96P double mutant TIM shows the enzyme-bound DHAP intermediate at lower energy than the enzyme-bound enediol, the energies of these two intermediates are poorly determined. In fact, the energy of the enzyme-bound enediol is determined from the K_i for PGH, and since PGH has no removable protons, it may better mimic the substrate than the intermediate in this case. Thus, the most likely explanation for the observation of an enzymic carbonyl group that can only reasonably be assigned to the protonated form of D165 is that the enzyme−enediol intermediate is a stable species in the E165D·S96P double mutant.

Structural Basis for the Improved Catalytic Activity of E165D·S96P TIM. The structural differences between the E165D single mutant isomerase and the E165D·S96P pseudorevertant isomerase are found in the subtle changes in water molecules bound at the active site of the enzyme. Two of these water molecules are present near the D165 side chain in the single mutant and are absent in the E165D·S96P pseudorevertant. The absence of these water molecules is expected to increase the hydrophobicity of the reactive site pocket near the catalytic base. This increase in the hydrophobicity of the environment would likely increase the pK_a of the catalytic base, thus making it more effective in removing the substrate protons even from a greater distance. The effect of solvent hydrophobicity on the pK_a's of carboxylic acids is a well-studied phenomenon; for example, the pK_a of acetic acid rises from 4.76 in 100% water to 10.14 in 82% dioxane−18% water (Hendrickson et al., 1970). It is interesting that an increase in hydrophobicity of the active site near position 165 does not improve the activity of the wild-type isomerase; in fact, the S96P single mutant has a catalytic efficiency that is reduced by 20-fold from the wild-type isomerase.

A new water molecule is present in the E165D·S96P pseudorevertant. This water molecule is located on the opposite face of the O−N−C−O plane of the PGH intermediate analog from the catalytic base. The distance from the water molecule to the two oxygens is short, and this water molecule could easily hydrogen-bond to the substrate or enediolate intermediates in the catalytic reaction. In this case, since the substrate is neutral and it becomes ionized upon proton removal, the effect of the hydrophobicity of the pocket is expected to be opposite that for the D165 side chain. Thus, this new water molecule probably decreases the pK_a of the substrate protons by increasing the hydrophilicity of the active site pocket. The altered location of active site water molecules may change the pK_a's of the enzymatic base and of the substrate. The loss of the two waters near the base, expected to increase the pK_a of the base, and the gain of the water molecule underneath the substrate, expected to decrease the pK_a of the substrate, would improve the catalytic activity of this mutant. It has always been puzzling that the pK_a of the glutamic acid base is approximately 7 and that of the substrate is approximately 20, and thus there is such a difference between the two. The argument has been made that proximity makes up for large pK_a differences in enzyme catalysis. It is thus very suggestive that to improve the catalytic activity of the E165D single mutant, for which the lesion was a loss of proximity, the pseudorevertant overcomes the proximity problem by better balancing the pK_a difference. 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" (Albery & Knowles, 1976).

The combination of differences in bound water molecules has a secondary effect discovered by the observance of a protonated carboxyl group in a nonpolar environment that could be assigned to the catalytic base, D165. The observation of a protonated D165 strongly suggests that, in the mutant enzyme, a reaction intermediate is stable relative to the enzyme-bound substrate and product ground states. The unique stability of this intermediate can be attributed to the combination of increased hydrophobicity of the active site pocket near D165 and the presence of a new water molecule capable of protonating the enediolate intermediate. By increasing the stability of the enediol intermediate, the activation free energy of the catalytic steps is further lowered by a "differential binding" effect (Albery & Knowles, 1976).

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