Electrophilic Catalysis in Triosephosphate Isomerase: The Role of Histidine-95[†,‡]

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ABSTRACT: Electrophilic catalysis by histidine-95 in triosephosphate isomerase has been probed by using Fourier transform infrared spectroscopy and X-ray crystallography. The carbonyl stretching frequency of dihydroxyacetone phosphate bound to the wild-type enzyme is known to be 19 cm^{-1} lower (at 1713 cm^{-1}) than that of dihydroxyacetone phosphate free in solution (at 1732 cm^{-1}), and this decrease in stretching frequency has been ascribed to an enzymic electrophile that polarizes the substrate carbonyl group toward the transition state for the enolization. Infrared spectra of substrate bound to two site-directed mutants of yeast triosephosphate isomerase in which histidine-95 has been changed to glutamine or to asparagine show *unperturbed* carbonyl stretching frequencies between 1732 and 1742 cm^{-1}. The lack of carbonyl polarization when histidine-95 is removed suggests that *histidine-95 is indeed the catalytic electrophile*, at least for dihydroxyacetone phosphate. Kinetic studies of the glutamine mutant (H95Q) have shown that the enzyme follows a subtly different mechanism of proton transfers involving only a single acid–base catalytic group. These findings suggest an additional role for histidine-95 as a general acid–base catalyst in the wild-type enzyme. The X-ray crystal structure of the H95Q mutant with an intermediate analogue, phosphoglycolohydroxamate, bound at the active site has been solved to 2.8-Å resolution, and this structure clearly implicates glutamate-165, the catalytic base in the wild-type isomerase, as the sole acid–base catalyst for the mutant enzyme. Glutamate-165 is the only residue that is significantly displaced in the H95Q mutant protein: the carboxylate group has moved by more than 2 Å from its position in the wild-type enzyme. The repositioning of glutamate-165 causes the carboxylate oxygen that is closer to the substrate to be almost equidistant from both C-1 and C-2 *and* both O-1 and O-2 of the substrate. The fact that glutamate-165 is recruited to perform the proton transfers involving the substrate oxygens during catalysis by the H95Q enzyme strongly suggests that histidine-95 is responsible for this function in the wild-type protein. Histidine-95 thus plays a dual role as an electrophile and as a general acid–base in the catalysis of triose phosphate isomerization.

Although the impressive catalytic efficiency of triosephosphate isomerase is well established, questions still remain about the structural features of the protein that make this enzyme such an excellent catalyst. Histidine-95 has been implicated in recent structural determinations as a possible electrophile and/or general acid–base catalyst. Electrophilic catalysis in enolization reactions such as those carried out by triosephosphate isomerase is common in organic reactions, but relatively few studies have demonstrated the direct involvement of electrophilic groups in enzymic reactions. One of the most powerful techniques for detecting electrophilic catalysis in reactions involving the enolization of carbonyl groups is Fourier transform infrared spectroscopy (FTIR). This method directly probes the carbonyl bond strengths in the ground state on a time scale ($\sim 10^{-13}$ s) that is short with respect to molecular motion. Ground-state destabilization, which is one way in which an enolization reaction could be accelerated, can thus readily be detected. Indeed, bond weakening due to substrate polarization on binding to the enzyme has been observed for several systems (Belasco & Knowles, 1980, 1983; Kurz & Drysdale, 1987). In wild-type triosephosphate isomerase (TIM), the majority of bound dihydroxyacetone phosphate (DHAP) has a carbonyl stretching frequency that is 19 cm^{-1} lower than that of DHAP free in solution. This shift strongly suggests that the enzyme distorts and destabilizes the ground state of the substrate toward the transition state of the enolization reaction by polarizing the carbonyl group. Until now, the identity of the enzymic functionality responsible for this polarization has remained uncertain, for crystallographic data (Banner et al., 1975; Alber et al., 1981; Davenport, 1986) had shown two possible candidates in the active site: lysine-13[1] and histidine-95.

In addition to its possible role as an electrophile in substrate polarization, histidine-95 might also function to mediate the proton transfers at oxygen in the reaction catalyzed by triosephosphate isomerase. The position of histidine-95 in the active site as determined by X-ray crystallography seems to be ideal for the role as a general acid–base. In the wild-type enzyme, the ϵ-nitrogen of the histidine's imidazole ring is 2.9 and 2.8 Å from the substrate oxygens on C-1 and C-2, re-

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[‡] The coordinates for the H95Q mutant of yeast triosephosphate isomerase have been submitted to the Brookhaven Protein Data Bank.

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[1] The residue numbering of the chicken enzyme is used. Compared with the chicken isomerase, the yeast enzyme has a deletion at position 2 and an insertion at position 57.

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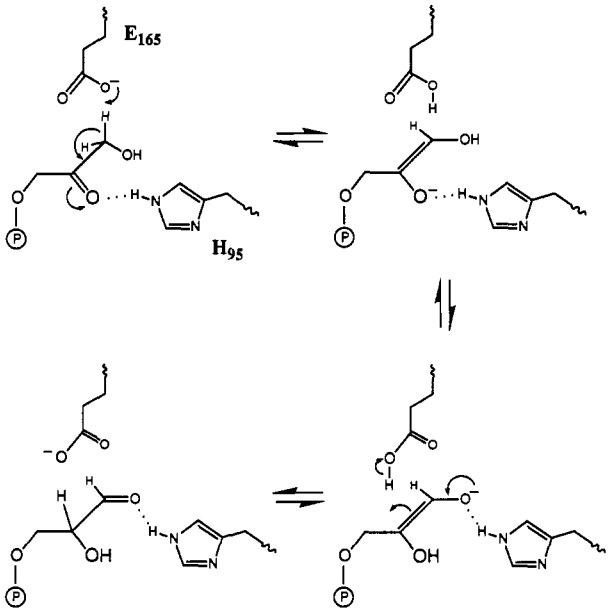
Scheme I: Mechanism of Wild-Type Triosephosphate Isomerase[a]



[a] The enzyme's catalytic groups are glutamate-165 (E_{165}) and histidine-95 (H_{95}).

spectively, and is thus in an excellent position to shuttle protons between these two centers (Scheme I). A hint that histidine-95 may indeed be the required proton transfer catalyst was suggested by the kinetic behavior of a site-directed mutant of the enzyme in which histidine-95 was changed to glutamine (H95Q). The mechanism of proton transfers catalyzed by this mutant enzyme is different from that followed by the wild-type isomerase and is consistent with catalysis being mediated by a single base that both removes the protons from the substrate carbon atoms and replaces them on the substrate oxygens (Scheme II). The kinetic data obtained for this mutant enzyme did not, however, rule out other mechanistic possibilities, such as the slow exchange of water molecules trapped at the active site or the presence of sequestered enzymic bases that cannot exchange protons (in their conjugate acid forms) with the medium (Nickbarg et al., 1988).

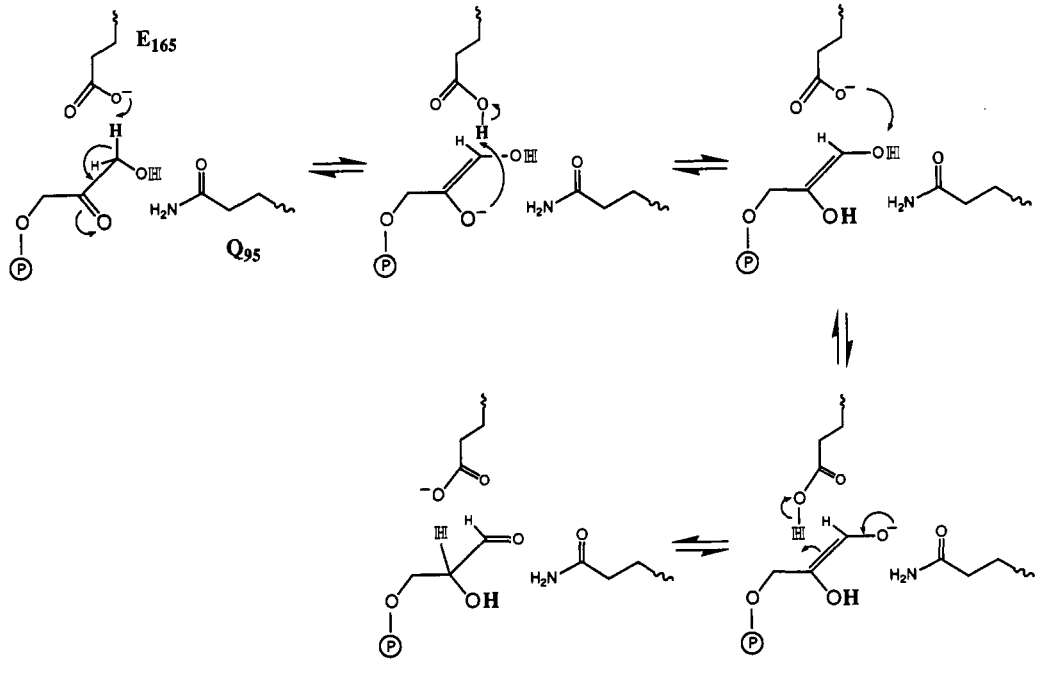
Site-directed mutagenesis is a powerful method for determining the functional role of particular amino acid residues in an enzymic reaction. In the simplest analysis, the residue of interest is changed to one that lacks the suspected function, and the behavior of the resulting protein is evaluated. In the case of histidine-95, two conservative changes have been made within the confines of the twenty naturally occurring amino acids, by replacing this residue with glutamine or with asparagine. Glutamine can in principle mimic the hydrogen bonding capacity of the ϵ-nitrogen of the imidazole ring of a histidine residue, and asparagine may replace the δ-nitrogen analogously. While neither glutamine nor asparagine is expected to have the proton transfer capability of histidine, each may exert a similar polar effect and occupy a roughly similar region of space. We report here an investigation of the H95Q and the H95N mutants of triosephosphate isomerase by FTIR spectroscopy to determine whether histidine-95 functions as the catalytic electrophile in the wild-type enzyme. In the case of the H95Q mutant, we also present the structure of the enzyme as determined by X-ray crystallography.

Materials and Methods

Reagents. Dihydroxyacetone phosphate, DL-glyceraldehyde 3-phosphate diethyl acetal, glucuronolactone, histidine, streptomycin, ampicillin, reduced nicotinamide adenine dinucleotide, DEAE-Sephadex A-25, Dowex-50W (H^+ form), and QAE-Sephadex A-120 were purchased from Sigma Chemical Co. (St. Louis, MO). Premixed Luria broth was from Gibco (Grand Island, NY) and prepared according to the manufacturer's instructions. Deuterium oxide (99.9% labeled) was purchased from MSD Isotopes (Montreal, Canada). All other reagents were from commercial sources and were used without further purification.

Substrates. DL-[1-^{13}C]Glyceraldehyde 3-phosphate was prepared by the method of Serianni et al. (1979) as modified by Belasco and Knowles (1983), further modified as specified

Scheme II: Mechanism of the H95Q Mutant Triosephosphate Isomerase Proposed by Nickbarg et al. (1988)[a]



[a] The enzyme's catalytic groups are glutamate-165 (E_{165}) and glutamine-95 (Q_{95}). The pathways of proton transfer (from C-1 to O-2 and from O-1 to C-2) are illustrated with a filled H and an open H, respectively.

below. Glycerol 1-phosphate was oxidized with $Pb(OAc)_4$ to glycolaldehyde phosphate, which was then allowed to react with [^{13}C]KCN (99% atom percent excess, Cambridge Isotope Labs, Cambridge, MA). The resulting cyanohydrin was reduced by catalytic hydrogenation over $Pd/BaSO_4$ and the product hydrolyzed to the aldehyde. The crude product was purified by chromatography on DEAE-Sephadex A-25 in 50 mM NaOAc buffer, pH 4.5. The purified product was concentrated by lyophilization, adjusted to a concentration of 270 mM in D-[1-^{13}C]glyceraldehyde 3-phosphate as determined by coupled assay (Nickbarg et al., 1988), and stored as a frozen solution in small portions at −70 °C. The solution also contained 270 mM L-[1-^{13}C]glyceraldehyde 3-phosphate, but this substrate does not bind to triosephosphate isomerase and is hydrated at neutral pH, so it is not observed in the FTIR. Each portion was thawed and then neutralized with NaOH (2 N) immediately before use.

[U-^{13}C]Dihydroxyacetone phosphate was prepared by the method of Belasco and Knowles (1983), modified as described below. [U-^{13}C]Glucose (Cambridge Isotope Labs) was first converted into [U-^{13}C]fructose 1,6-bisphosphate by hexokinase/ATP, phosphoglucose isomerase, and phosphofructokinase/ATP. The [U-^{13}C]fructose 1,6-bisphosphate was cleaved with aldolase and triosephosphate isomerase to yield an equilibrium mixture (1:22) of D-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). The mixture of triose phosphates was then converted to the lithium salt by using a column of Dowex 50W (Li^+ form). The solution was lyophilized, and the triose phosphates were dissolved in a volume of water so as to give a final concentration of 270 mM. Small portions of this solution were stored frozen at −70 °C. The pH of the aqueous solution of the lithium salts of the triose phosphates was neutral.

Proteins. Triosephosphate isomerase genes from yeast were subcloned into a derivative of pBS +/− (Stratagene, La Jolla, CA) that has been described previously (Blacklow & Knowles, 1990). This phagemid vector allowed the efficient production of single-stranded DNA for site-directed mutagenesis. This phagemid contains, on an *Eco*RI-to-*Pst*I fragment, the *trc* promoter upstream from the complete gene for yeast triosephosphate isomerase. The mutant in which histidine was changed to glutamine (H95Q) was constructed by P. Lodi (unpublished work). The mutant in which histidine was changed to asparagine (H95N) was constructed by using an oligonucleotide-directed mutagenesis kit (Amersham, Arlington Heights, IL) and a mutagenic primer having the sequence 5′-C GGA GTT ACC CAA AAT AAC C-3′, following the method of Eckstein (Nakamaye & Eckstein, 1986). The entire sequence of the mutant gene was determined to ensure that no other changes had been made. The genes for the mutant proteins were further subcloned into the high-expression vector pKK223-3 (Pharmacia, Piscataway, NJ) to avoid instabilities of the phagemid upon large-scale growth. The derived plasmids contain tandem promoters (*tac* from pKK223-3 and *trc* from the *Eco*RI-to-*Pst*I phagemid fragment; see above), which allowed for the production of approximately 50–80 mg of protein/L of cell culture. The expression vectors were used to transform *Escherichia coli* strain DF502, which is a strep^R, *tpi*$^-$ strain that was generously donated by D. Fraenkel and has been previously described (Straus & Gilbert, 1985).

To prepare isomerase samples, the appropriate bacterial transformants were grown in a final volume of 10 L in 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/L), L-histidine (80 mg/L), streptomycin (100 mg/L), and ampicillin (200 mg/L). The cells were harvested after 12–20 h by centrifugation at 3000*g*. The yield of cell paste was typically 50–70 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 contained more than 95% of the TIM activity. After overnight dialysis against TE buffer (10 mM Tris-HCl buffer, pH 7.8, containing 1 mM EDTA), the dialyzed protein was loaded onto a column (300 mL) of QAE-Sephadex A-120 equilibrated with TE buffer. The purified isomerases were eluted with a linear gradient (1 L plus 1 L) of KCl (0–300 mM) in the same buffer. When necessary, further purification was accomplished on a Q-Sepharose Hi Load column (Pharmacia, Piscataway, NJ) with the same gradient. Final protein yields from a 10-L culture were 250–500 mg of purified enzyme, which was >99% homogeneous as assessed by denaturing polyacrylamide gel electrophoresis (Laemmli, 1970). The protein samples were concentrated by using Centripep and Centricon concentrators (Amicon, Danvers, MA). Steady-state kinetic constants (k_{cat} and K_m) were determined in both the forward and reverse directions as described previously (Nickbarg & Knowles, 1988).

FTIR Spectroscopy. Spectra were obtained by 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 was prepared by lyophilizing 100 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA, and then dissolving the solid 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 about 60 μL. The Circle cell was exhaustively washed with the buffer between each filling with sample and cleaned daily with a dilute solution of low-foaming detergent and washed extensively with H_2O. Protein (70 μL, 10 mN final concentration of active sites) was mixed with substrate (2 μL, 7 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 values of ν_{max}, since the bandwidths of the absorptions for free substrate and free enzyme are so much larger than those for the enzyme-bound ligands.

X-ray Crystallography. Protein crystals were grown in 100-μL portions containing TIM (20 mg/mL) and phosphoglycolohydroxamate (1.5 mM) in 200 mM Tris-HCl buffer, pH 6.8, containing 2-mercaptoethanol (1 mM) and EDTA (1 mM). Poly(ethylene glycol) 4000 was added until the protein just precipitated, and a small amount of buffer was then added to clarify the solution. The resulting solutions were allowed to stand at room temperature in half-dram vials with cork stoppers until crystals appeared (5–10 days). Diffraction data indicated that these crystals belong to the space group $P2_1$ and are isomorphous to those of the wild-type enzyme complexed to phosphoglycolohydroxamate (Alber et al., 1981).

Table I: Steady-State Kinetic Constants[a] for H95Q and H95N Mutant Triosephosphate Isomerases

	k_{cat}^+ (s^{-1})	K_m^+ (mM)	K_i^b (mM)	k_{cat}^- (s^{-1})	K_m^- (mM)	k_{cat}^+/K_m^{+c}
wild type	1100	3.6	14	2900	0.56	1
H95Q	5.3	3.0	15	23	0.70	0.006
H95N	4.8	5.8	44	32	3.2	0.003

[a] k_{cat}^+ and K_m^+ relate to DHAP as substrate, k_{cat}^- and K_m^- relate to GAP as substrate. [b] For the competitive inhibitor arsenate. [c] Relative to the wild-type enzyme.

One crystal (0.5 mm × 0.4 mm × 0.3 mm) was mounted at room temperature in a quartz capillary tube with mother liquor on either side to prevent the crystal from drying out. The unit cell of the crystal was found to be a = 74.27 Å, b = 83.73 Å, c = 38.96 Å, and β = 99.94° from least-squares centering of 23 orientation reflections on a Rigaku AFC5 diffractometer. A complete data set to 2.8 Å was measured by using Ni-filtered Cu Kα X-rays produced from a Rigaku rotating anode operating at 60 kV and 200 mA. The data were collected by the full integration method, scanning on ω for a total of 1.4°. The peak of each reflection was counted for 7.0 s while its background, which was collected 1.4° away from the calculated peak position, was counted for 3.5 s. Those reflections that had structure factor magnitudes less than 10 standard deviations were measured twice. The intensities of four standard reflections, which were distributed through reciprocal space, were measured every 300 reflections in order to correct for radiation damage. At the end of data collection, the intensities of the standard reflections had decreased linearly by about 40%.

After intensity data were corrected for absorption (North et al., 1968) and radiation damage, 81% of the theoretical number of reflections had intensities greater than one standard deviation and were kept for further work. Rigid-body refinement of a model of the wild-type enzyme in which all solvent molecules were deleted and His-95 was replaced with glutamine resulted in an rms movement (relative to the wild-type enzyme) of about 1 Å for all non-hydrogen atoms. This rigid body movement lowered the R-factor from 47% to 21%. Subsequent refinement by simulated annealing according to the program X-PLOR (Brunger et al., 1987) lowered the R-factor to 18.4% for all 9635 reflections between 10 and 2.8 Å, with an rms deviation for the structure of 0.023 Å for bond lengths and 4.0° for bond angles. The coordinates for this mutant have been submitted to the Brookhaven Protein Data Bank.

RESULTS

Kinetics. The steady-state kinetic constants for two mutant yeast isomerases (H95Q and H95N) have been determined. Those for the H95Q mutant were reported previously (Nickbarg et al., 1988) and are listed with those for the H95N enzyme (from this work) in Table I. While the turnover rate for each of the mutant enzymes with DHAP is slower than that for wild-type TIM, the enzymes still retain significant catalytic activity. Considering that the wild-type enzyme catalyzes the isomerization reaction some 10^{10} fold faster than does a nonenzymic base of pK_a 7 (Richard, 1984), these mutants are still very effective catalysts.

If the chemical steps for the interconversion of the enzyme·substrate and enzyme·product complex are much slower than the rates of binding and release of substrate and product from the active site, then the steady-state kinetic constants (k_{cat} and K_m) can be used to calculate a simplified three-step free energy profile in which the chemical interconversion is represented by the central step. Under these circumstances, K_m values represent the dissociation constants of the enzyme·substrate and enzyme·product complexes, and k_{cat} values represent the rates of the conflated steps for the interconversion of these complexes. In constructing the free energy profiles, we assume that the "on" rates for substrate and product are the same as that for the wild-type enzyme (4×10^8 M^{-1} s^{-1}: Albery & Knowles, 1976) and that the standard state is 40 μM [the actual concentration of triose phosphates in vivo; see Albery and Knowles (1976)]. This method of illustrating and comparing kinetic data has been described more fully elsewhere (Straus et al., 1985). The free energy profiles for the two mutant enzymes derived in this manner are compared to that for the wild-type enzyme in Figures 1 and 2. It is clear from these figures that the transition state(s) for proton abstraction are of much higher free energy for the mutant enzymes than for the wild type and that the two mutant enzymes do not bind the substrates quite as tightly as does the wild-type isomerase. These kinetic results are entirely consistent with the removal of an enzymic electrophile that contributes to catalysis but is not absolutely essential for catalytic activity. Such an electrophilic group would presumably also contribute

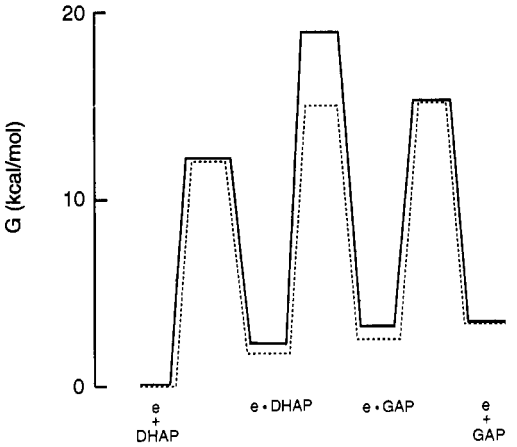


FIGURE 1: Gibbs free energy profile for the simplifed (three-step) reaction path for the H95Q mutant triosephosphate isomerase (solid lines) compared to the profile for the wild-type isomerase (dotted lines). Values of ΔG were determined from the rate constants reported in Table I, according to Straus et al. (1985). A standard state of 40 μM has been assumed. The "on" rate for both substrates has been assumed to be the same as for the wild-type enzyme, at 4×10^8 M^{-1} s^{-1}. e, enzyme; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate. For details, see the text.

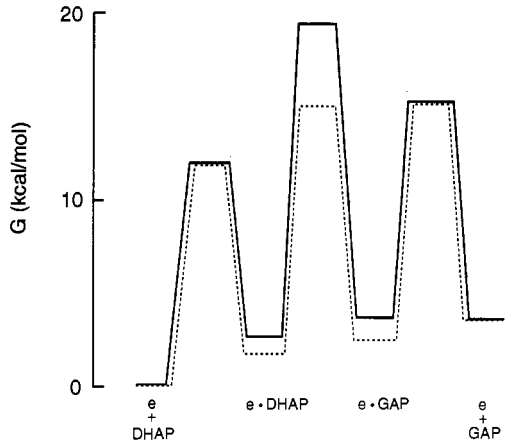


FIGURE 2: Gibbs free energy profile for the simplified (three-step) reaction path for the H95N mutant triosephosphate isomerase (solid lines) compared to the profile for the wild-type isomerase (dotted lines). Conditions were as described for Figure 1.

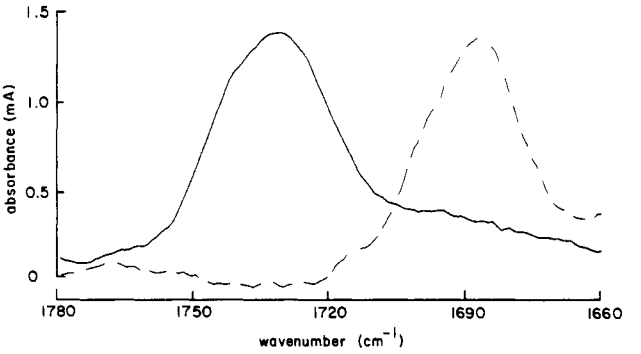


FIGURE 3: Fourier transform infrared spectra of DHAP (7 mM) and of [U-^{13}C]DHAP (7 mM) is deuterated 100 mM Tris-HCl buffer, pD 7.8. For the conditions, see the text.

something to substrate binding and thus affect the values of K_m, as is observed (see Table I).

FTIR Spectroscopy. The FTIR spectrum of each of the mutant isomerases was determined in the presence of unlabeled and of ^{13}C-labeled substrates. The substrate concentration was 7 mM and the enzyme concentration was 10 mN (in active sites). The fact that the K_m values for DHAP with the mutant enzymes are 3–6 mM means that, under the conditions used, about 50–65% of the substrate is enzyme bound (the nonideality of the solutions at such high enzyme concentrations makes these estimates only approximate). Accordingly, the appropriate levels of absorbance of free substrate and free enzyme have been subtracted from the observed spectra (Figures 4–6). For spectra taken in D_2O, spectral subtractions can be made in the region above 1700 cm^{-1}. Below 1700 cm^{-1}, the protein's carbonyl groups absorb too strongly to allow reliable subtractions to be made. Since enzyme-bound substrates reach equilibrium rapidly, the bound triose phosphates are present at concentrations determined by the internal equilibrium constant (that is, by the ratio of enzyme·substrate complex to enzyme·product complex).

Labeling of a carbonyl carbon with ^{13}C causes the carbonyl group stretching frequency to drop by about 40 cm^{-1} due to the effect on the reduced mass. The FTIR spectra of DHAP and of [U-^{13}C]DHAP are overlaid in Figure 3 and show that, in this case, a shift of 43 cm^{-1} is observed on isotopic substitution. Such shifts are useful for identifying the carbonyl groups that correspond to particular absorbances in the spectra of enzyme-bound species. Normally, labeling with ^{13}C moves the carbonyl absorbance below 1700 cm^{-1} into the region that is overwhelmed by protein amide absorbances. The use of triose phosphates labeled with ^{13}C, either at C-1 or at all three carbon atoms, allows the identification of particular absorbances as deriving from DHAP or from GAP, since either the GAP carbonyl absorbance only (for the [1-^{13}C] material) or both the GAP and DHAP carbonyl absorbances (for the [U-^{13}C] material) are thereby shifted below 1700 cm^{-1}.

Figure 4 shows the FTIR spectra of substrate bound to the wild-type isomerase. Two peaks are seen, one at 1732 cm^{-1} and one at 1713 cm^{-1}. In the spectrum of the enzyme with 1-^{13}C-substrates neither of these peaks is shifted, and it is therefore clear that both the absorbances are due to the carbonyl of DHAP since neither responds to 1-^{13}C labeling. This conclusion (from ^{13}C-labeled substrates) confirms that reached earlier for the chicken enzyme on the basis of substrates labeled with ^{18}O and with ^{2}H (Belasco & Knowles, 1980). The observation of a major band due to enzyme-bound DHAP at 1713 cm^{-1} indicates that the wild-type isomerase polarizes this substrate's carbonyl group toward the transition state for the reaction [that is, toward the transition state for

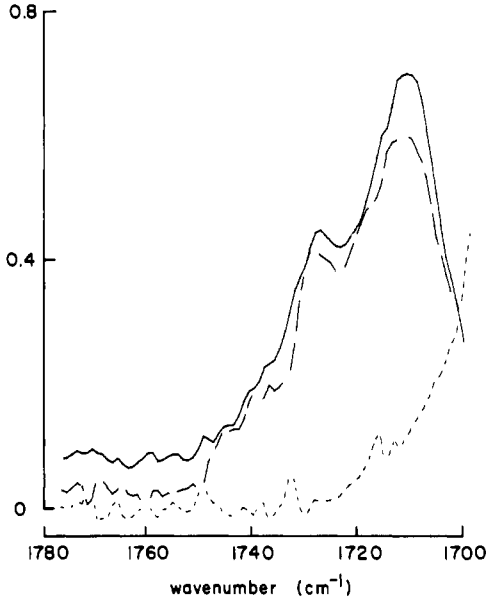


FIGURE 4: Fourier transform infrared spectra of wild-type yeast triosephosphate isomerase (10 mN) in the presence of substrate (7 mM). Solid line, isotopically unlabeled substrates; dashed line, 1-^{13}C-labeled substrates; dotted line, U-^{13}C-labeled substrates. For the conditions, see the text.

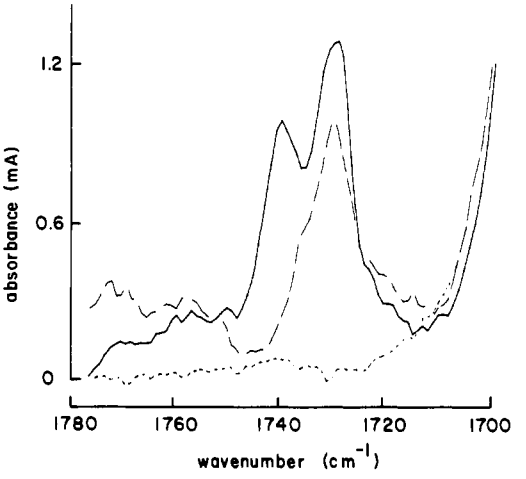


FIGURE 5: Fourier transform infrared spectra of the H95Q mutant yeast triosephosphate isomerase (10 mN) in the presence of bound substrate (7 mM). Solid line, isotopically unlabeled substrates; dashed line, 1-^{13}C-labeled substrates; dotted line, U-^{13}C-labeled substrates. For the conditions, see the text.

the enolization of DHAP to the enediol(ate) intermediate]. The second peak at 1732 cm^{-1} has been attributed to DHAP bound nonproductively (Belasco & Knowles, 1980). The fact that no absorbances are seen for GAP is due either to the internal equilibrium constant largely favoring DHAP or to the polarization of this carbonyl group being so strong that its absorption falls below 1700 cm^{-1}. No decision between these possibilities can be made at this time. The absorbance bands due to DHAP bound to the enzyme seen in Figure 4 are considerably narrower than that for DHAP free in solution (Figure 3). This finding is consistent with the narrowing of substrate infrared absorbances upon binding to an enzyme surface that is usually observed and that is presumed to derive from restricted rotation and the more defined environment of the active site compared with aqueous solution (Fisher et al., 1980).

Figure 5 shows the infrared spectra of the H95Q mutant enzyme containing bound substrates, both unlabeled and la-

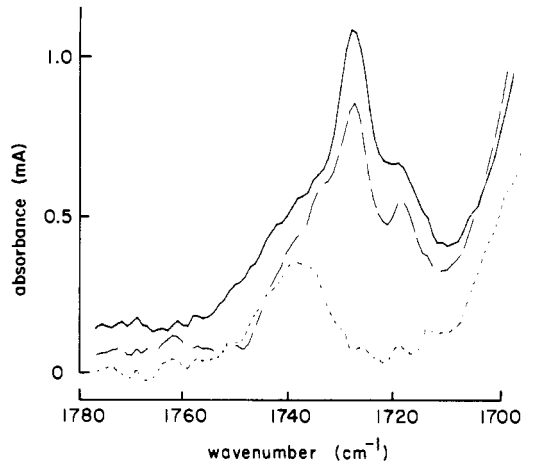


FIGURE 6: Fourier transform infrared spectra of the H95N mutant yeast triosephosphate isomerase (10 mN) in the presence of bound substrate (7 mM). Solid line, isotopically unlabeled substrates; dashed line, 1-^{13}C-labeled substrates; dotted line, U-^{13}C-labeled substrates. For the conditions, see the text.

beled with ^{13}C. It is clear from these data that the peak at 1743 cm^{-1} is due to the carbonyl group of GAP and that the peak at 1733 cm^{-1} is due to that of DHAP. The featureless baseline (from 1780 to 1710 cm^{-1}) observed when [U-^{13}C]-triose phosphates are used, shows that for this mutant, no *enzyme* carbonyl groups are perturbed by substrate binding. The width at half-height for the bands observed in Figure 5 is less than half that observed for DHAP free in solution. This observation confirms the conclusion that the absorbing species observed in the spectra of substrates bound to the H95Q mutant isomerase are indeed enzyme bound. Furthermore, although the peaks overlap and cannot be integrated reliably, the areas corresponding to bound DHAP (at 1733 cm^{-1}) and to bound GAP (at 1743 cm^{-1}) correlate approximately with the ratio of liganded forms of the enzyme (E·DHAP:E·GAP) of 3, calculated from the steady-state kinetic constants (Table I). [For a reaction where the interconversion rate of enzyme·substrate and enzyme·product complexes is slow and most of the substrates are enzyme bound, the ratio of enzyme·substrate:enzyme·product is approximated by k_{cat}^-/k_{cat}^+.]

It is obvious from a comparison of the spectra shown in Figure 5 with those of the wild-type isomerase (Figure 4) that *the substrate carbonyls are not polarized by the mutant H95Q enzyme*. This result is strengthened by the infrared spectra observed for the H95N mutant shown in Figure 6. In this case, the steady-state kinetic constants predict an internal equilibrium constant of about 12 in favor of enzyme-bound DHAP, suggesting that we should not expect to see an absorbance band for bound GAP. Consistent with this expectation, the spectrum of unlabeled enzyme-bound substrates is essentially the same as that for 1-^{13}C-labeled enzyme-bound substrates. No absorbance from GAP is present, and it is evident that the major absorbance at 1733 cm^{-1} is due to enzyme-bound DHAP. Since when all the substrate carbons are labeled with ^{13}C the absorbance at 1743 cm^{-1} remains, this band must be due to an *enzymic* carbonyl group. Such a perturbation of an enzyme carbonyl group upon substrate binding has been observed previously (Belasco & Knowles, 1983).

X-ray Crystallography. Structural data for the H95Q mutant TIM with phosphoglycolohydroxamate bound at the active site have been obtained to 2.8-Å resolution. In order to assess the error in the coordinate positions of this structure, the two identical subunits in the asymmetric unit were superimposed. While the identical sequence for the two subunits might suggest that they should have identical structures, errors and different crystallographic environments may lead to different local conformations. The rms difference between the two subunits when all non-hydrogen atoms are superimposed is 0.75 Å. From this comparison, we estimate the error in this structure to be on the order of 0.75 Å.

The active-site structure of the wild-type enzyme with bound phosphoglycolohydroxamate and the corresponding view of the liganded H95Q mutant isomerase are shown in Figure 7, panels A and B, respectively. A comparison of these two structures reveals a surprising result (Figure 7C). The protein backbone and the side chains of the two enzymes are almost superimposable except for the region near glutamate-165. Remarkably few other differences are observed between the two structures, the residue displacements all being less than 1 Å. The carboxylate group of glutamate-165, however, has swung out toward the solvent. When the two subunits are independently superimposed on the wild-type structure on the basis of the orientation matrix for all atoms, the three atoms of the carboxylate group are all more than 2 Å away from their position in the wild-type structure.

Discussion

Electrophilic Catalysis. Triosephosphate isomerase catalyzes the interconversion of DHAP and GAP so efficiently that the transition states for the chemical steps of the reaction [the enolization of each of the substrates to form the enediol(ate) intermediate] are not kinetically significant. Several pieces of evidence suggest that one of the ways in which the isomerase increases the rate of these chemical steps is by electrophilic catalysis. Thus it was shown that the rate of reduction of the DHAP carbonyl group by borohydride is 8-fold faster when the DHAP is bound to triosephosphate isomerase than when it is free in solution (Webb & Knowles, 1974). This acceleration, which more than compensates for the presumed lower steric accessibility of the substrate when bound, is most readily interpreted in terms of a polarization of the substrate's carbonyl group by the enzyme. Fourier transform infrared spectra of DHAP bound to the wild-type enzyme subsequently confirmed the substantial polarization of the carbonyl group of this substrate, although some of the enzyme-bound material appeared to be bound nonproductively. None of these early experiments, however, allowed any identification of the enzymic group or groups responsible for substrate polarization and for electrophilic catalysis. In fact, inspection of the crystal structure of the liganded enzyme suggested that polarization of the substrate carbonyl groups could have derived from interaction of the carbonyl oxygen with lysine-13 and/or histidine-95 or from the partial enolization of the substrate by the interaction of glutamate-165 with (for DHAP) the *pro-R* proton on C-1. Site-directed mutagenesis is a powerful technique for probing the function of particular amino acid residues, and this approach allows individual residues to be altered and the behavior of the resulting mutant proteins to be determined. In the present case, histidine-95 has been changed to either glutamine or asparagine in an effort to define the catalytic role of this residue. The mutant proteins have been analyzed kinetically, by FTIR spectroscopy, and by X-ray crystallography.

The three enzymic groups at the active site of triosephosphate isomerase that might account for the polarization of the substrate that is effected by this enzyme are histidine-95, lysine-13, and glutamate-165. The last two, lysine-13 and glutamate-165, are retained in the H95Q and H95N mutants described here. The fact that infrared spectra of substrate bound to each of these mutants show that the substrate's

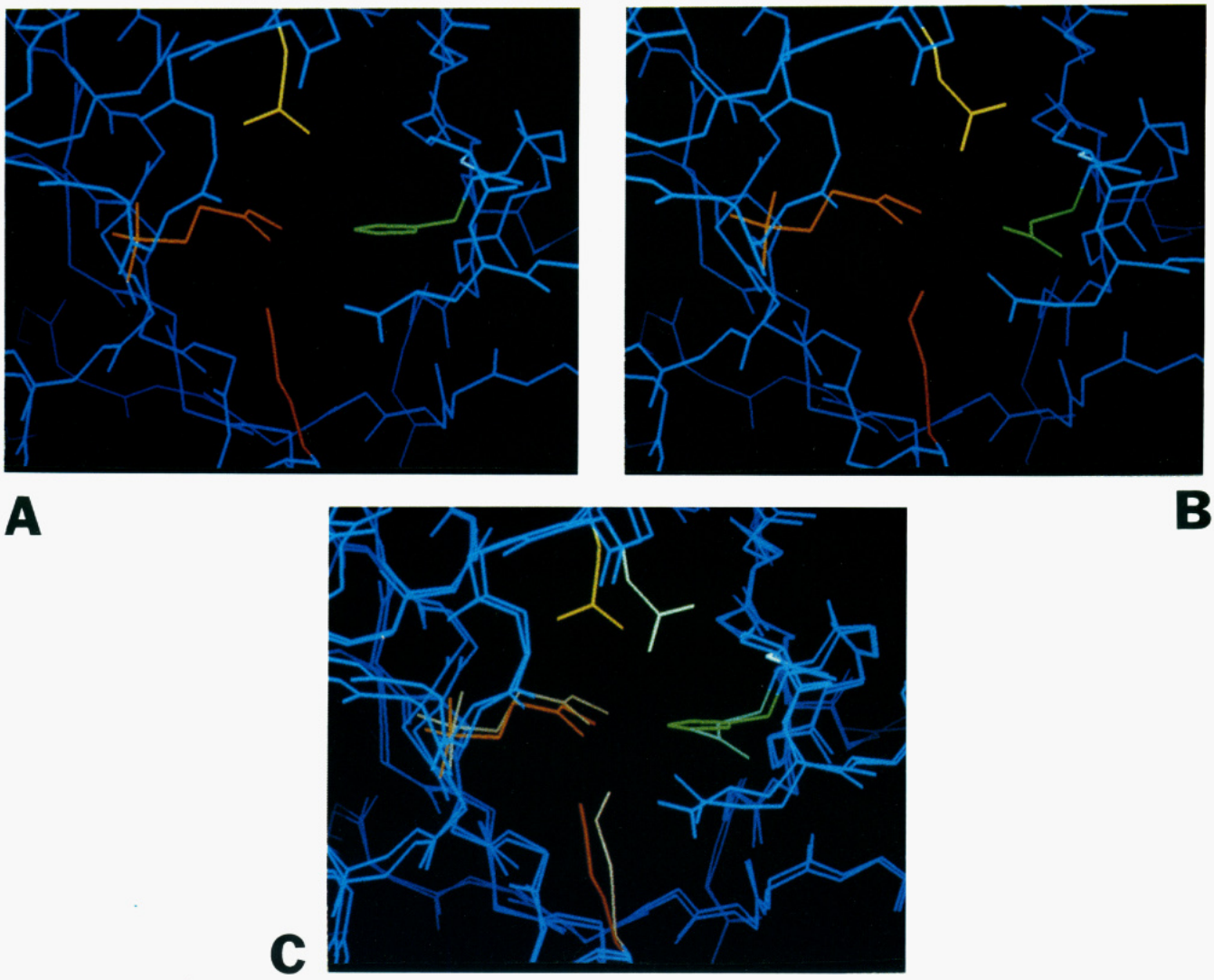


FIGURE 7: Structure of the active-site region of wild-type triosephosphate isomerase with bound phosphoglycolohydroxamate (A) and the same view of the active site of the H95Q mutant isomerase with bound phosphoglycolohydroxamate (B). The residue colors for these two views are as follows: lysine-13, red; histidine-95 or glutamine-95, green; serine-96 (–NH– group), pink; glutamate-165, yellow; phosphoglycolohydroxamate, orange. In (C) is shown an overlay of the structures in (A) and (B). In this overlay, the intensity of the colors of the H95Q mutant isomerase residues is faded relative to those of the wild-type enzyme.

carbonyl groups are *un*polarized (in contrast to the polarization seen with the wild-type enzyme) strongly suggests that neither lysine-13 nor glutamate-165 significantly affects substrate polarity. Although it might be argued that changing the active-site histidine to another amino acid results in a loss of the polarizing interaction whatever its origins, analysis of several other isomerase mutants (such as E165D and the double mutants H95N/S96P and E165D/S96P) show that these proteins polarize the bound substrates as well or nearly as well as the wild type. In fact, histidine-95 appears to account for *all* of the substrate carbonyl polarization that is observed by infrared spectroscopy and, therefore, for all of the electrophilic catalysis. One might wonder if a glutamine at position 95 could retain some of the hydrogen-bonding character of histidine-95 and thus still interact with the substrate carbonyl groups. However, the amide nitrogen of glutamine-95 does not occupy precisely the same position as the ϵ-nitrogen of histidine-95. While the distances from the histidine ϵ-nitrogen to the substrate oxygens on C-1 and C-2 in the wild-type enzyme are 2.8 and 2.9 Å, the distances from the glutamine ϵ-amino group to the substrate oxygens in the mutant enzyme are 3.2 and 4.0 Å. The lesser catalytic effectiveness of the H95Q mutant enzyme should not, therefore, surprise us.

Proton Transfer. The first evidence that histidine-95 plays a role in proton transfer was obtained from the unusual kinetic behavior of the H95Q mutant isomerase (Nickbarg et al., 1988). Tritium exchange and transfer experiments demonstrated that while more than 3% of the protons removed from C-1 of DHAP appear at C-2 of GAP in the reaction catalyzed by the wild-type enzyme, no such transfer of protons is observed in the reaction catalyzed by the H95Q mutant. This result implies a rapid equilibrium of substrate protons with the medium, and yet it was found that no solvent-derived protons appear in either substrate after partial reaction. To accommodate these findings, we proposed that a *single* enzymic base abstracts the proton from C-1 of DHAP and places it on O-2 of the first-formed enediolate intermediate (Nickbarg et al., 1988). The same base then removes the proton from O-1 of the enediol before protonating C-2 to produce the product GAP. These transfers, which are required by the tritium transfer data reported by Nickbarg et al. (1988), are outlined in the pathway shown in Scheme II. This scheme accounts for the near absence of substrate-to-product proton transfer observed with the mutant enzyme, because the proton that ends up on C-2 of GAP is always a solvent-derived proton that comes from O-1, a solvent-exchangeable position. It is

Table II: Distances from the Closer Oxygen of Glutamate-165 to Substrate Atoms and to the Main-Chain NH Group of Serine-96[a]

	substrate atom				serine-96
	O-1	O-2	C-1	C-2	NH
wild type	3.6	4.4	2.8	3.4	5.4
H95Q mutant	2.8	3.2	3.0	3.2	2.9

[a] Distances are given in angstroms.

not surprising that little exchange with external solvent protons into remaining substrate is observed in this scheme, since the proton on the H95Q enzyme's catalytic base (glutamate-165) will have little chance to exchange with solvent protons before its rapid capture by the highly basic enediolate intermediate.

The most likely candidate for the single base required by Scheme II is glutamate-165. The crystal structure of the wild-type enzyme shows that this side chain is positioned over the substrate and could, if it moved by about 2 Å, also reach the protons on the substrate oxygens. That this occurs is strongly suggested by the crystal structure of the H95Q mutant isomerase. The structure of the main chain and side chains of this mutant protein can be almost exactly superimposed on those of the wild-type structure, with the exception of glutamate-165 (Figure 7). The carboxylate of this residue has moved about 2 Å compared to its position in wild-type TIM, and in the mutant enzyme it comes much closer to both substrate oxygens. The distances listed in Table II were determined from the crystal structures of the enzyme containing phosphoglycolohydroxamate bound at the active site, this intermediate analogue providing a good approximation of the location of bound substrate. The distances tabulated are from the closer oxygen of glutamate-165 to the carbons (C-1 and C-2) and oxygens (linked to C-1 and C-2) of the substrate. It is apparent from these data that, in the H95Q mutant protein, the carboxylate of glutamate-165 is within proton transfer distance of *both carbons and both oxygens* of the substrate. This result lends strong support to the mechanism proposed by Nickbarg et al. (1988) for the H95Q mutant enzyme (Scheme II). The tritium transfer results (Nickbarg et al., 1988) require a single catalytic base, and the crystal structure presented here identifies this base as the carboxylate of glutamate-165. This body of evidence notwithstanding, it is still somewhat surprising that a catalytic residue (glutamate-165), which is already responsible for one function in the reaction catalyzed by the wild-type enzyme, can perform an added function in the mutant H95Q protein without a very severe effect on the catalytic efficiency.

Structural Information. Several questions remain to be answered about the catalysis mediated by the H95Q mutant isomerase. First, what are the *structural* features that cause glutamate-165 to swing out in the H95Q enzyme when no other active-site residues have moved significantly? Inspection of the structures of the wild-type enzyme and the H95Q mutant suggests the answer to this question. In the wild-type enzyme (Figure 7A), glutamate-165 lies above the "plane" of the substrate (defined by O-1, C-1, C-2, and O-2), poised to abstract the proton from C-1 of dihydroxyacetone phosphate or from C-2 of glyceraldehyde phosphate. One oxygen of glutamate-165 is 3.4 and 2.8 Å from the substrate's C-2 and C-1, respectively, and the other oxygen is 3.6 and 3.1 Å from C-2 and C-1, respectively. Histidine-95 in the wild-type enzyme can form a strong hydrogen bond either to O-1 (2.9 Å) or to O-2 (2.8 Å) of the substrate and thus provide the electrophilic component of catalysis.

In the structure of the H95Q mutant (Figure 7B), there are just two major changes: glutamine-95 replaces histidine-95, and glutamate-165 swings out toward the solvent. The crystal structure suggests that two new hydrogen bonds hold the glutamate side chain in its new position: one oxygen of glutamate forms a hydrogen bond to the main-chain NH of serine-96 (2.9 Å) and the other oxygen to the amino function of the carboxamido side chain of glutamine-95 (3.1 Å). These new interactions define the position of the catalytic base (glutamate-165) in the H95Q mutant, which results in the mechanistic behavior observed for this enzyme. The reason why the H95Q change effects this move of the carboxylate base of glutamate-165 appears to be because in the wild-type enzyme histidine can act (with its ϵ-nitrogen) only as a *single* hydrogen-bond donor (to the substrate), whereas in H95Q glutamine can act (with the NH_2 group of its carboxamido side chain) as a *double* hydrogen-bond donor (to the substrate and to the carboxylate of glutamate-165). These relationships are evident from the crystal structures of the two enzymes and nicely accommodate the kinetic and spectroscopic behavior of these isomerases.

The second interesting difference between the structures of wild-type TIM and the H95Q mutant is that, in the former, glutamate-165 is positioned such that it is a syn orbital of the carboxylate that appears to be used to abstract the substrates' carbon-bound protons, whereas the closer orbital of the glutamate-165 carboxylate in the H95Q mutant is an anti orbital. Gandour (1981) has pointed out that the basicity of the anti orbital of a carboxylate group is less than that of the syn orbital and suggests that this could be worth some 100–1000-fold in catalytic power. Although experimental confirmation of this proposal has not yet been made [an effect of about 10-fold having been achieved by Tadayoni et al. (1989), while Cramer and Zimmerman (1990) conclude that "the catalytic benefit of a syn carboxylate is minimal"], it seems possible that some of the loss in catalytic power of the H95Q mutant comes from the fact that the mutant enzyme uses the less basic anti orbital of glutamate-165. Whichever orbital of the carboxylate is used, however, most of the loss of catalytic potency of the H95Q isomerase must derive from the fact that glutamine is a much poorer electrophile than the imidazole ring of histidine,[2] which is reflected in the lesser polarization of bound substrate carbonyl groups.

We have shown that histidine-95 is the enzymic electrophilic that contributes to the catalysis mediated by triosephosphate isomerase. This residue appears also to function as a proton transfer catalyst in the interconversion of the two enediolate intermediates in the reaction. The relatively conservative changes of this histidine to either glutamine or asparagine result in the complete loss of substrate carbonyl polarization, and we can conclude that histidine-95 is solely responsible for this function in the wild-type enzyme. Histidine-95 provides a strong single hydrogen bond to the substrates' carbonyl oxygens, and this interaction results in the observed carbonyl polarization and contributes to catalysis of the enolization. The role of histidine-95 in effecting proton transfer between the two intermediate enediolates is suggested by kinetic experiments in which a change in mechanism is observed for the H95Q mutant isomerase. The crystal structure of this enzyme is entirely consistent with the observed single-base mechanism in which glutamate-165 does double duty and takes over the proton transfers to and from the substrate oxygens as well as those to and from the substrate carbons. This remarkable recruitment of a catalytic residue to perform an added function

[2] The pK_a of imidazole is about 14 (Yagil, 1967) and that of simple amides is around 18 [Molday and Kallen (1972) and see Gilbert and Jencks (1979)].

is an impressive example of opportunism in enzyme catalysis.

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Calcineurin-Catalyzed Reaction with Phosphite and Phosphate Esters of Tyrosine†

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ABSTRACT: A convenient synthesis is reported for the preparation of the phosphite ester of tyrosine methyl ester. By use of calcineurin, at 30 °C, a phosphite ester was hydrolyzed with a V_M value [119 nmol/(min·μg of E)] approximately 500 times greater than that obtained with tyrosine phosphate [0.23 nmol/(min·μg of E)] as substrate, but with similar K_M values (12 mM for Tyr-PH ME, 11 mM for Tyr-P). Acid phosphatase, on the other hand, hydrolyzed the phosphite ester with a V_M and K_M value lower than those obtained with tyrosyl phosphate. The temperature dependence of the kinetic parameters (K_M and V_M) was evaluated, and the activation parameters were obtained with both substrates. The entropy of activation associated with the enzymatic hydrolysis of tyrosine phosphate agrees with the entropy change for the hydrolysis of the monoanion of phosphate monoesters. The energy of activation for both substrates was in agreement with the energy change for hydrolysis of the oxygen–phosphorous linkage of phosphate monoester monoanions and phosphite esters. These results are consistent with a scheme of general acid catalysis in the action of calcineurin.

Enzyme-catalyzed protein phosphorylation–dephoshorylation is an important regulatory mechanism (Nimmo & Cohen, 1977; Krebs & Beavo, 1979). In the last several years, much attention has focused upon the tyrosyl phosphorylation of cellular proteins by specific tyrosyl kinases and the dephosphorylation of phosphotyrosyl residues by tyrosyl protein phosphatases. Tyrosyl phosphorylation is associated with the regulation of cellular activities such as proliferation, differentiation, and transformation (Hunter & Sefton, 1982; Heldin & Westermark, 1984; Sefton & Hunter, 1984; Swarup et al., 1984; Sefton, 1985; Coughlin et al., 1988). It has also been suggested that increased cellular phosphorylation of protein tyrosyl residues plays an important role in the regulation of growth processes (Hunter & Cooper, 1983).

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