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Neutral Imidazole Is the Electrophile in the Reaction Catalyzed by Triosephosphate Isomerase: Structural Origins and Catalytic Implications[†,‡]

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ABSTRACT: To illuminate the role of histidine-95 in the catalytic reaction mediated by triosephosphate isomerase, ^{13}C and ^{15}N NMR titration studies have been carried out both on the wild-type enzyme and on a mutant isomerase in which the single remaining histidine (that at the active site) has been isotopically enriched in the imidazole ring. ^{15}N NMR has proved especially useful in the unambiguous demonstration that the imidazole ring of histidine-95 is uncharged over the entire pH range of isomerase activity, between pH 5 and pH 9.9. The results require that the first pK_a of histidine-95 is below 4.5. This abnormally low pK_a rules out the traditional view that the positively charged imidazolium cation of histidine-95 donates a proton to the developing charge on the substrate's carbonyl oxygen. ^{15}N NMR experiments on the enzyme in the presence of the reaction intermediate analogue phosphoglycolohydroxamate show the presence of a strong hydrogen bond between $N^{\epsilon 2}$ of histidine-95 and the bound inhibitor. These findings indicate that, in the catalyzed reaction, proton abstraction from C-1 of dihydroxyacetone phosphate first yields an enediol*ate* intermediate that is strongly hydrogen bonded to the neutral imidazole side chain of histidine-95. The imidazole proton involved in this hydrogen bond then protonates the enediolate, with the transient formation of the enediol-imidazol*ate* ion pair. Abstraction of the hydroxyl proton on O-1 now produces the other enediolate intermediate, which collapses to give the product glyceraldehyde 3-phosphate. This initially surprising sequence is more reasonable when it is recognized that the pK_a values of the enediol and the perturbed pK_a[2] of the imidazole ring of histidine-95 may be rather close to each other, allowing for two facile and rapid proton transfers that interconvert the two enediolates. To our knowledge, this is the first reported example of the participation of an imidazolate side chain in an enzyme-catalyzed reaction. The imidazole ring of histidine-95 lies at the amino terminus of a short α-helix that will, in accord with what is known from the behavior of substituted imidazoles in solution, lower both the first and the second pK_a values of the side chain of histidine-95.

Triosephosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of (*R*)-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). As shown in Figure 1, glutamate-165 is the catalytic base responsible for proton abstraction from carbon (Waley et al., 1970; Hartman, 1971; de la Mare et al., 1972; Banner et al., 1975; Lolis et al., 1990), while histidine-95 is believed to be the catalytic acid (Belasco & Knowles, 1980; Nickbarg et al., 1988; Komives et al., 1991). The results of Rieder and Rose (1959) and of Bloom and Topper (1956) are consistent with the intermediacy of an enediol(ate) in the catalyzed reaction (Figure 1).

The role of histidine-95 in the catalytic mechanism of triosephosphate isomerase has been the subject of considerable study and speculation (Belasco & Knowles, 1980; Komives et al., 1991; Nickbarg et al., 1988). There are at least three functions that could be performed by this residue. First, histidine-95 may polarize the carbonyl group in the substrate DHAP, facilitating the abstraction of the *pro-R* hydrogen at C-1 by glutamate-165. Earlier studies by Belasco and Knowles (1980) demonstrated that the carbonyl group of DHAP is indeed polarized on binding to the enzyme, and Komives et al. (1991) have recently shown that histidine-95 is responsible for this polarization in the wild-type enzyme. The substrate is oriented in the active site so that the *pro-R* hydrogen is perpendicular to the plane defined by O-1, C-1, C-2, and O-2,

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[‡]This paper is respectfully dedicated to Professor Robert H. Abeles on the occasion of his 65th birthday.

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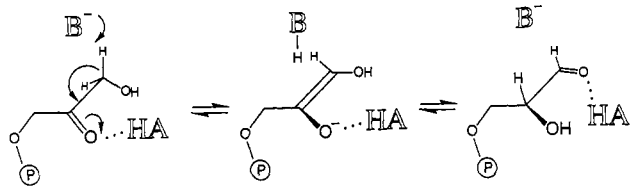

FIGURE 1: An overview of the reaction catalyzed by triosephosphate isomerase. B^- is the catalytic base (glutamate-165), and HA is the catalytic acid (histidine-95).

thus exploiting the known stereoelectronic advantage for proton abstraction to form an enol(ate) (Corey & Sneen, 1956). Second, crystallographic data indicate that histidine-95 is ideally positioned to stabilize the proposed reaction intermediate: the $N^{\epsilon 2}$ of the imidazole ring of this residue is within 2.9 Å of both O-1 and O-2 of the substrate, and this center is very likely involved in hydrogen bonding with the substrates and reaction intermediate(s). The third possible function of histidine-95 is to aid in the necessary transfer of protons to and from O-1 and O-2 of the enediol(ate) intermediate before this collapses to product. Nickbarg et al. (1988) found that when histidine-95 is replaced by glutamine, glutamate-165 acts as *both* a catalytic acid and a catalytic base. This result implies that histidine-95 functions as an acid during catalysis by the wild-type enzyme. The focus of the present work is to define the role of histidine-95 in the reaction catalyzed by triosephosphate isomerase, using results from ^{13}C and ^{15}N NMR spectroscopy of enzyme samples in which this amino acid has been uniquely labeled with the appropriate isotope.

The primary point of uncertainty concerning histidine-95 is its protonation state in the active enzyme. On the one hand, several arguments can be advanced in favor of a protonated histidine side chain. Chemical precedent suggests that an appropriately positioned imidazolium ring (as in Figure 2A) would polarize the substrate's carbonyl group more strongly than neutral imidazole (see Figure 2B) and result in more effective catalysis of the enolization. The pK_a of an imidazolium group is near 7, making it a much stronger acid than imidazole, the pK_a of which is slightly above 14 (Yagil, 1967). Since the infrared stretching frequency of the carbonyl group of DHAP shifts 19 cm^{-1} on binding to the enzyme (Belasco & Knowles, 1980), it is clear that the enzyme polarizes this group considerably more strongly than does solvent water (the pK_a of which is 15.7). Other facts consistent with a charged imidazolium ring for histidine-95 include the tight binding of the inhibitor phosphoglycolate as its trianion (Campbell et al., 1978). Moreover, the very much weaker binding of phosphoglycolate to the mutant H95Q enzyme (Nickbarg et al., 1988) suggests that something more than merely a hydrogen-bond interaction between the carboxylate group of the inhibitor and the imidazole ring is lost when histidine is replaced by glutamine. Yet despite the mechanistic attractiveness of an imidazolium group as a general acid catalyst for the enzyme, there are countervailing arguments, the strongest of which comes from X-ray crystallographic data. In the structure of both the unliganded and the liganded enzyme, $N^{\delta 1}$ of histidine-95 is within hydrogen-bonding distance of the main-chain NH of glutamate-97 (Lolis et al., 1990; Lolis & Petsko, 1990). If $N^{\delta 1}$ acts as a hydrogen bond acceptor, it cannot, of course, become protonated without breaking the hydrogen bond. The second argument for an uncharged histidine is that the heterocyclic imidazole ring lies at the positive end of a short helix (from residues 95 to 102: Figure 3) that is expected to *lower* the pK_a of the histidine from its unperturbed value near 7 (Hol, 1985). Finally, it can be argued that, with neutral imidazole as the general acid, the first-formed intermediate would be an enedio*late*, a species that is reasonably simulated by the trianionic form of phosphoglycolate, which is known to bind tightly to the enzyme (Campbell et al., 1978).

Several groups (Witanowski et al., 1972; Blomberg et al., 1977; Bachovchin & Roberts, 1978; Alei et al., 1980; Roberts et al., 1982) have studied the nitrogen NMR of imidazole and substituted imidazoles and have shown that the protonation state of the imidazole ring and, even, the hydrogen-bonding characteristics of each nitrogen can be determined with striking

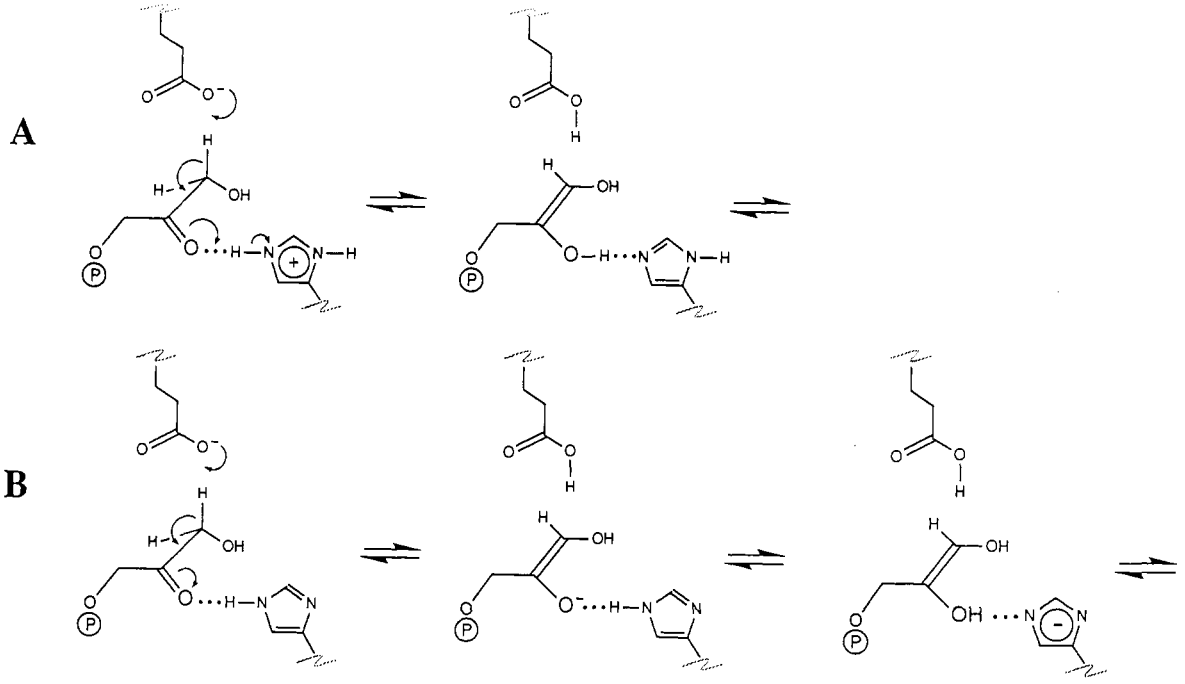

FIGURE 2: Two possible roles for histidine-95 in the mechanism of triosephosphate isomerase. Mechanism A involves the imidazolium of histidine-95 that acts as a general acid, initially forming an imidazole–enediol pair. Mechanism B involves the neutral imidazole of histidine-95 that acts as a general acid, initially forming an imidazole–endiol*ate* pair that (to accommodate the remaining proton movements) may transiently form an imidazol*ate*–enediol pair.

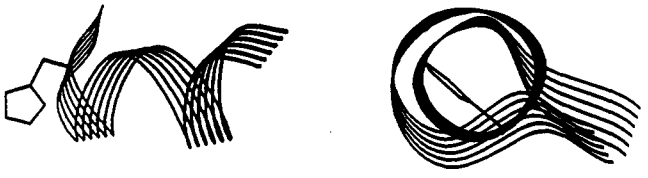

FIGURE 3: Two views of histidine-95 and the active site α-helix that is formed by residues 95–102 (from the coordinates of the wild-type yeast enzyme in the presence of phosphoglycolohydroxamate; Davenport et al., 1991).

clarity by this technique. Bachovchin (1986) has elegantly exploited the capabilities of ^{15}N NMR to illuminate the nature of the catalytic triad of α-lytic protease. He has also used the magic angle spinning method to show that the active site histidine in α-lytic protease has a pK_a that is different in the crystalline enzyme and in solution (Smith et al., 1989). This result has highlighted the caution that must be exercised when crystallographic data are used to infer the protonation state and hydrogen-bonding arrangements of the amino acid side chains of proteins in solution. We have therefore investigated histidine-95 in triosephosphate isomerase by ^{15}N NMR, the results from which allow a clear determination both of the protonation state of the imidazole ring and of the hydrogen-bonding characteristics of each of its nitrogens.

Experimental Procedures

Materials. pBS vectors were obtained from Stratagene (La Jolla, CA), pKK223-3 vectors were obtained from Pharmacia (Piscataway, NJ), and M13mp18 phage was obtained from New England Biolabs (Beverly, MA). Oligonucleotide-directed mutagenesis kits were obtained from Amersham (Arlington Heights, IL). The pH electrode was from Microelectrodes, Inc. (Londonderry, NH).

Escherichia coli strain DF502 was the generous gift of D. Fraenkel and has been previously described (Straus & Gilbert, 1985). DF502 is strep^R, *tpi*$^-$, and *his*$^-$.

Histidine specifically labeled with ^{13}C at the $C^{\epsilon 1}$ position (99 atom % excess) was obtained from Merck Sharp & Dohme (Montreal, Canada); histidine specifically labeled with ^{15}N at the $N^{\delta 1}$ position (99 atom % excess) was obtained from ICON (Summit, NJ); histidine specifically labeled with ^{15}N at both the $N^{\delta 1}$ and $N^{\epsilon 2}$ positions (99 atom % excess) was obtained both from ICON and from the Los Alamos National Laboratory (Los Alamos, NM). The inhibitor phosphoglycolate was obtained from Sigma (St. Louis, MO), and the inhibitor phosphoglycolohydroxamate was a gift from Dr. J. Belasco.

Reagents for cell growth, and for purification and assay of the enzyme, were as described by Komives et al. (1991).

Methods. The gene encoding triosephosphate isomerase from Baker's yeast was obtained (from E. Lolis) as an insert in M13mp18 phage. This gene was modified so that it could be subcloned into a derivative of the pBS expression vector and then further subcloned into the high-expression vector pKK223-3: site-directed mutations were introduced into the M13 DNA by using oligonucleotide-directed mutagenesis. To subclone the gene into a modified pBS expression vector, an *Nco*I site was introduced at the 5′ end of the gene and a *Pst*I site was added beyond the 3′ end of the gene. To create the *Nco*1 site, the following mutagenic primer was used: 5′-CTAGCCATGGTTAGTTTATG-3′. To add the *Pst*1 site, the mutagenic primer 5′-GCTTTCTGCAGTCATCAAT-3′ was used. All mutagenesis procedures followed the methods of Eckstein (Nakamaye & Eckstein, 1986). The wild-type gene was subcloned from M13mp18 into a derivative of pBS+ using the new *Nco*1/*Pst*1 sites and was further subcloned into a slightly modified pKK223-3. The pKK223-3 vector that resulted from the subcloning contained tandem promoters (*tac* from pKK223-3, and *trc* from the *Eco*R1-to-*Pst*1 gene fragment from pBS+). In the ^{13}C NMR experiments with wild-type protein this expression system was used to provide the isotopically labeled protein. In all the ^{15}N NMR experiments as well as in the later ^{13}C NMR experiments, an H103Q·H185Q mutant isomerase was used. In ^{13}C experiments with denatured enzyme the wild-type isomerase was used. To change histidine-103 to glutamine, the mutagenic primer 5′-CATCTTCTTGGAAGTAAG-3′ was used. To change histidine-185 to glutamine, the mutagenic primer 5′-GGA-AGCTTGAATATCTTG-3′ was used. These mutations were made while the gene remained in M13mp18. The entire gene was sequenced and then subcloned into the modified pKK223-3 vector as described above.

[^{13}C]Histidine and [^{15}N]Histidine Incorporation. The appropriate expression vectors were used to transform *E. coli* strain DF502. These vectors allowed for the production of 40–70 mg of triosephosphate isomerase/L of culture; the volume of liquid culture used to grow the DF502 varied from 4 to 10 L depending on the amount of enzyme desired. The best results in terms of the yield of isomerase were obtained under the following conditions. To each 1 L of M-63 minimal salts (Miller, 1972) were added glucuronolactone (0.4% w/v), glycerol (0.04% v/v), $MgSO_4$ (1 mM), thiamin hydrochloride (1 mg/L), ampicillin (200 mg/L), and streptomycin (100 mg/L). All amino acids except histidine were dissolved in water in approximately the same proportions as in Casaminoacids, and the solution was sterile-filtered. This amino acid supplement was added to the medium such that the final concentration of each of the 19 amino acids was about 1.6 g/L. Appropriately labeled histidine was then added in the amount of 30 mg/L ([$^{13}C^{\epsilon 1}$]-(*R,S*)-histidine for ^{13}C experiments involving wild-type isomerase or the H103Q·H185Q double mutant; or [$^{15}N^{\delta 1}$,$^{15}N^{\epsilon 2}$]-(*R,S*)-histidine for H103Q·H185Q preparations; or [$^{15}N^{\delta 1}$-(*R,S*)-histidine in the H103Q·H185Q preparation). An inoculum of cell culture (about 4 mL of culture/L from a fresh, 100-mL overnight growth) was then added. The cells were grown at 37 °C and harvested by centrifugation at 5000*g* for 15 min after 12–20 h of growth.

Purification of Isotopically Labeled Enzyme. Cells were suspended at 4 °C in 10 mM Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) and 2-mercaptoethanol (1 mM) and then lysed by using a continuous-flow French pressure cell (Aminco, Urbana, IL) at 20000 psi. The resulting suspension was centrifuged at 16000*g* for 1 h. The supernatant was diluted in 10 mM Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) and 2-mercaptoethanol (1 mM), and the purification continued as follows. Finely ground ammonium sulfate was added to the extract while stirring at 4 °C until the solution reached 40% saturation with ammonium sulfate. The resulting solution was stirred for 2 h at 4 °C and then centrifuged at 16000*g* for 1 h, and the pellet was discarded. Ammonium sulfate was added to the supernatant with stirring until 55% saturation was reached. This solution was then stirred for 2 h at 4 °C and then centrifuged for 1 h at 16000*g*. Ammonium sulfate was added to the supernatant until 90% saturation with ammonium sulfate was reached. This solution was stirred for 2 h at 4 °C and then centrifuged for 1 h at 16000*g*. The pellet from the 55–90% fraction was then resuspended in 10 mM Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) and 2-mercaptoethanol (1 mM), and then dialyzed in 10 mM Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) and 2-

mercaptoethanol (1 mM), before purification on a column of either Mono-Q 10/10 or Q-Sepharose Hi-Load (Pharmacia, Piscataway, NJ). Buffer A was 10 mM Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) and 2-mercaptoethanol (1 mM). A gradient of buffer B (0–30%) was used to elute the isomerase (buffer B is buffer A containing 500 mM KCl). Final isomerase yields were about 20 mg/L culture of ~95% pure protein as determined by protein assays, activity assays, and denaturing polyacrylamide gel electrophoresis (Laemmli, 1970).

Determination of Kinetic Constants. Steady-state kinetic constants for H103Q·H185Q yeast triosephosphate isomerase were determined at 30 °C and found to be almost identical with those for the wild-type enzyme. The K_m value for GAP is 0.93 mM (compared to 1.5 mM for wild type), the K_m value for DHAP is 2.9 mM (compared to 2.3 mM for wild type; Nickbarg & Knowles, 1988), and the specific activity is 14000 units/mg (compared to 14900 units/mg for the wild type).

Isomerase activity assays were carried out as follows. To 1 mL of assay buffer [100 mM triethanolamine hydrochloride pH 7.6, containing EDTA (10 mM)] at 30 °C were added NADH (140 μg) from a stock solution in water, GAP (100 μL of a 35–55 mM solution, prepared as described below), glycerol-3-phosphate dehydrogenase (5 μL, free from triosephosphate isomerase activity, prepared as described below), and a sample of isomerase (5 μL of a solution containing about 10^{-4} mg/mL).

The GAP solution was prepared by adding glyceraldehyde 3-phosphate diethyl acetal (monobarium salt) (200 mg), Dowex 50 (H^+ form) (3 mL), and H_2O (3 mL) to a Falcon tube (Becton Dickinson, Lincoln Park, NJ). The mixture was shaken until the GAP diethyl acetal dissolved, and the tube was then immersed in boiling water, with shaking, for 3 min. The tube was placed on ice to cool and then centrifuged at 2000*g* for 10 min. The supernatant was removed and H_2O (2 mL) was added. The mixture was then clarified by centrifugation and the supernatant recovered.

Glycerol-3-phosphate dehydrogenase free from triosephosphate isomerase was prepared by adding bromohydroxyacetone phosphate (about 10 equiv over the amount of isomerase present; obtained from E. Komives) to a suspension of the dehydrogenase (about 150 μL of a solution of 10 μg/μL). This mixture was incubated at 4 °C for 1 h. Remaining bromohydroxyacetone phosphate was then removed by ultrafiltration [using a Centricon filtration unit (Amicon, Danvers, MA)] from added 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (5 mM) and 2-mercaptoethanol (1 mM).

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were recorded at 125.759 MHz on a Bruker AM500N spectrometer, with a 5-mm standard probe equipped to detect ^1H, ^{13}C, ^{15}N, and ^{31}P. The ^{13}C NMR samples were 0.3–3 mN in enzyme active sites and were prepared by concentration of enzyme in a Centricon filtration unit. The pH of the sample was changed by washing the enzyme in the Centricon unit with one of the following solutions (in D_2O): 20–40 mM sodium acetate, 20–40 mM Tris-HCl, or 20–40 mM sodium pyrophosphate. On rare occasions, dilute aqueous NaOD or dilute aqueous DCl was added to an enzyme sample before concentration to obtain the desired pH. The pH of the sample was measured with a glass combination electrode, and values reported are the meter readings that are not corrected for D_2O. Chemical shifts are reported relative to internal (trimethylsilyl)propionic acid (at about 2 mg/mL) and are uncorrected for the small effect of pH on the standard (De Marco, 1977). Spectra were acquired at room temperature by using a 30° radio frequency pulse with a spectral width of about 30000 Hz and 16K data points. A relaxation delay of 0.05–0.1 s was used.

The pH of the sample was measured before and after spectral data accumulation, and the data were not used if the pH of the sample had changed by more than 0.05 pH unit. Denaturation of the enzyme was shown by activity assay to be negligible (less than 10%) under the conditions of accumulation. When ^{13}C spectra were taken of enzyme in the presence of either of the inhibitors, phosphoglycolohydroxamate or phosphoglycolate, the inhibitor concentration in the sample was between 3 and 4 mM.

^{15}N NMR Spectroscopy. ^{15}N NMR spectra were recorded at 50.697 MHz on a Bruker AM500N spectrometer, with a 5-mm standard probe equipped to detect ^1H, ^{13}C, ^{15}N, and ^{31}P. The ^{15}N samples were 1–4 mN in enzyme active sites and were prepared as described above. Samples were made up in aqueous D_2O (10% v/v) and referenced against external ^{15}N histidine that itself had been referenced to 1 M HNO_3 at 0 ppm. [It has been reported by Bachovchin (1986) that the ^{15}N chemical shifts of positively charged histidine are 200.5 and 202.9, relative to 1 M HNO_3. Positive shifts are upfield from the 1 M HNO_3 standard.] The peaks on the external reference of [$^{15}N^{\delta1}$,$^{15}N^{\epsilon2}$]-D,L-histidine (62 mM, pulse width 18°, spectral width 10000 Hz, relaxation delay 0.9 s) were set to these values, and the spectral reference value thus obtained was used to reference the spectrum. Spectra of the enzyme were acquired at room temperature with a 90° pulse, a spectral width of 10000 Hz, 16K or 32K data points, and a relaxation delay of 0.9 s. The decoupler frequency was 9100 Hz for most spectra, although it was found that a decoupler frequency of 13000 Hz gave higher quality spectra when the inhibitor phosphoglycolohydroxamate was bound. Typically, spectra were processed with 20-Hz line broadening. Under the conditions of accumulation the samples retain at least 85% of their original catalytic activity. When ^{15}N spectra were taken of the isomerase in the presence of either of the inhibitors phosphoglycolohydroxamate or phosphoglycolate, the inhibitor concentration in the sample was 10 mM.

Results and Discussion

^{13}C NMR. In our first experiments, we investigated the titration behavior of the three histidines (95, 103, and 185) of yeast triosephosphate isomerase using ^{13}C NMR spectroscopy. The bacterial strain DF502 produces no endogenous isomerase and is a histidine auxotroph. When this strain is transformed with pKK223-3 carrying the triosephosphate isomerase gene and isotopically labeled histidine is added to the growth medium, the enzyme is expressed at high levels and all of its histidines bear the isotopic label. It has been well documented that the ^{13}C resonances of the imidazole ring of histidine experience changes in chemical shift with changes in protonation state (Reynolds et al., 1973; Deslauriers et al., 1974; Richarz & Wüthrich, 1978; Otvos & Browne, 1980). The $C^{\epsilon1}$ carbon has a chemical shift that changes by as much as 2.6 ppm on protonation, and this carbon is covalently bound to a hydrogen so that it relaxes rapidly and may also show a nuclear Overhauser enhancement. When [$^{13}C^{\epsilon1}$]histidine was used to label the protein, three resonances were observed in the ^{13}C NMR spectrum of the purified isomerase, and the dependence of the chemical shift of each of these resonances on pH was determined. One of the three resonances indicates a histidine in the enzyme that titrates between 136.5 and 138.8 ppm with a pK_a of 7.46 (Figure 4A). In contrast, the resonances from the other two histidines, at 135.4 ppm and at 138.1 ppm, are essentially invariant with pH over a wide range

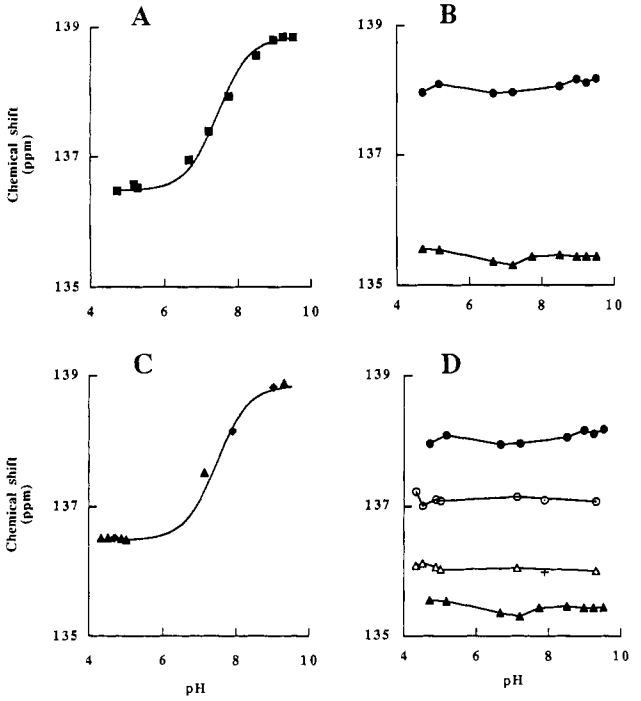

FIGURE 4: ^{13}C NMR chemical shift versus pH of ^{13}C-enriched wild-type triosephosphate isomerase. (A) Titration curve of histidine-103 (■) for the unliganded enzyme. The curve is theoretical for a pK_a of 7.46. (B) Chemical shift of histidine-95 (●) and histidine-185 (▲) as a function of pH for the unliganded enzyme. (C) Titration curve of histidine-103 for the unliganded enzyme [from (A)], with the experimental chemical shifts for the enzyme in the presence of either phosphoglycolohydroxamate (▲) or phosphoglycolate (◆). (D) Chemical shift of histidine-95 in the unliganded enzyme (●); histidine-95 in the enzyme that is liganded with either phosphoglycolohydroxamate (○) or phosphoglycolate (⊙); histidine-185 in the enzyme that is liganded with either phosphoglycolohydroxamate (△) or phosphoglycolate (+); and histidine-185 in the unliganded enzyme (▲), as a function of pH. All these ^{13}C spectra were taken in D_2O (95–99% v/v). For details, see Experimental Procedures.

(Figure 4B). The protonation state of these two histidine residues evidently does not change between pH 4.3 and pH 9.5. It thus appears that the pK_a value of each of these histidines is either above 10 or below 4 and that each has been markedly perturbed from the expected value near 7 (Richarz & Wüthrich, 1978; preliminary results indicate that in the denatured isomerase all three histidines titrate with pK_a values between 6.5 and 6.7). On the basis of studies with short peptides, the ^{13}C chemical shift of $C^{\epsilon 1}$ of an uncharged ring of a nonterminal histidine residue is around 137.8 ppm (relative to tetramethylsilane), while that of $C^{\epsilon 1}$ of a positively charged imidazolium side chain is about 135.2 ppm (Richarz & Wüthrich, 1978). These data therefore suggest that the nontitrating peak at 135.4 ppm derives from a histidine residue that is positively charged throughout the pH range investigated and that the peak at 138.1 ppm comes from a histidine residue that remains neutral over this pH range. These indications notwithstanding, the evident perturbation in the pK_a values of these two histidines makes it likely that each of these side chains is in an unusual environment on the enzyme. We must, therefore, be particularly cautious in making any statements about imidazole ring protonation state that are based only on the chemical shift of the $C^{\epsilon 1}$ peak.

In an effort to characterize the protonation state of the two *nontitrating* histidine residues unambiguously, the effect of inhibitor binding on the ^{13}C resonances was determined. The ^{13}C spectrum of the isomerase was therefore measured in the presence of the so-called transition-state analogue, phosphoglycolohydroxamate. This molecule binds tightly to the enzyme (K_i = 15 μM; Nickbarg & Knowles, 1988) and has been presumed to mimic the enediol or enediolate intermediate (Figure 1) of the catalyzed reaction (Collins, 1974). X-ray crystallographic data support this belief (Davenport et al., 1991), and we hoped that the perturbation of the ^{13}C histidine resonances would allow both the resonance assignment and the residue protonation state to be established. In the presence of bound phosphoglycolohydroxamate, the ^{13}C spectrum of the titrating histidine was essentially identical with that observed in the unliganded enzyme (Figure 4C). In contrast, the two nontitrating peaks were shifted to new, pH-independent positions at 136.1 and 137.1 ppm (Figure 4D). Thus, although the binding of the intermediate analogue has little or no effect on the titrating histidine, ligation perturbs the chemical shifts of the other two histidine residues, without moving their pK_a values into the observable range. Similar NMR experiments with the isomerase in the presence of the inhibitor phosphoglycolate gave analogous results (data included in Figure 4).

To determine which of the resonances in the ^{13}C NMR spectrum derives from the active site residue, histidine-95, we followed common practice and replaced histidine with glutamine. Although glutamine cannot act as a general acid, it can resemble histidine as a hydrogen bond donor, and one may hope that the three-dimensional structure of the enzyme is perturbed only minimally. Mutant isomerases were therefore prepared that contained glutamine at both positions 103 and 185. This double mutant protein, H103Q·H185Q, was isolated and found to be fully active, and to be kinetically indistinguishable from the wild-type enzyme. A ^{13}C NMR spectrum of the unliganded H103Q·H185Q mutant isomerase isolated from cells grown on [$^{13}C^{\epsilon 1}$]histidine indicated that the nontitrating peak at 138.1 ppm corresponds to histidine-95. This peak shifts to 137.1 ppm upon binding of the inhibitor phosphoglycolohydroxamate. ^{13}C NMR studies with an H103Q mutant isomerase then allowed us to complete the histidine assignments: the residue that titrates with a pK_a of 7.46 is histidine-103, and the residue that gives a nontitrating resonance at 135.4 ppm is histidine-185. Insofar as the chemical shift of the ^{13}C resonance is diagnostic, the resonance position of histidine-95 at 138.1 ppm suggests that this residue is uncharged between pH 4.3 and pH 9.5 and that there is a significant perturbation (of 1 ppm) on binding of the inhibitor. Yet the invariance of the ^{13}C resonance of histidine-95 with pH means that this statement rests only upon the value of the chemical shift, and it seemed imprudent to base any mechanistic conclusion on this single fact. It was decided, therefore, to obtain more detailed information from ^{15}N NMR spectroscopy.

^{15}N NMR. The great value of ^{15}N NMR in defining the protonation and hydrogen-bonding state of histidine residues in proteins was first demonstrated by Bachovchin and Roberts (1978) in their study of the titration behavior of the single histidine of α-lytic protease. Subsequently, Bachovchin has collated the chemical shifts of the $N^{\delta 1}$ and $N^{\epsilon 2}$ resonances of the imidazole side chain of histidine as a function of the protonation state of the ring and with the involvement of the nitrogen atoms in hydrogen bonds (Bachovchin, 1986). The data that are relevant to histidine residues in proteins are summarized in Table I. From this table it is clear that even though the resonance from a nitrogen atom that is involved in a hydrogen bond is shifted some 10 ppm (upfield for an acceptor, downfield for a donor), the ^{15}N NMR spectrum of a cationic imidazolium group shows two closely spaced signals around 201 ppm,[1] whereas neutral imidazole gives two sepa-

Table I: Typical ^{15}N Chemical Shift Values of Histidine Residues in Proteins[a]

nitrogen type	nucleus not involved in hydrogen bond[b] (ppm)	nucleus involved in hydrogen bond[b] (ppm)
N—H (type α)	210	200
N: (type β)	128	138
+N—H (type α+)	201	191

[a] Bachovchin (1986). All chemical shifts are relative to 1 M HNO_3 and are reported such that positive values are upfield from this standard. [b] Upon donation of a hydrogen bond, the resonance position of a histidyl nitrogen can shift as much as 10 ppm downfield; upon reception of a hydrogen bond, a histidyl nitrogen resonance can shift up to 10 ppm upfield.

rated nitrogen resonances, at about 210 and 128 ppm. It is thus clear that ^{15}N NMR is much more informative than the corresponding ^{13}C or ^{1}H spectra for defining the state of histidine side chains in proteins.

In the case of triosephosphate isomerase, we simplify the spectrum and allow the unambiguous assignment of resonances to the histidine of interest, histidine-95, by replacing all the other histidine residues with glutamine. This mutant H103Q·H185Q isomerase was then isolated from a *his*$^-$ auxotroph grown on doubly labeled [$^{15}N^{\delta 1}$,$^{15}N^{\epsilon 2}$]histidine. The kinetic parameters of the mutant enzyme were, as mentioned before, virtually identical with those of the wild type. ^{15}N NMR spectra were obtained from the unliganded HQ·HQ mutant enzyme, and the results are shown in Figure 5. As expected, there are two signals from the single remaining histidine (histidine-95): one corresponding to $N^{\delta 1}$ and one corresponding to $N^{\epsilon 2}$. The nominal pH of the solution from which this spectrum derives is pH 9.4, and from the chemical shifts of the two resonances, at 121.3 and 213.5 ppm, it is clear that the imidazole ring of histidine-95 is neutral under these conditions. To validate this observation and to discover the pK_a value of the imidazole group, ^{15}N spectra of the HQ·HQ isomerase were measured over the pH range from 4.9 to 9.9. As is evident from the inset to Figure 5, the positions of the two ^{15}N resonances are invariant with pH. These results show that histidine-95 in the unliganded yeast isomerase remains neutral throughout the pH range and confirm the tentative conclusion reached above on the basis of ^{13}C NMR studies. The sharpness of the signals suggests that the imidazole ring of histidine-95 remains predominantly in one tautomeric form. To assign the two nitrogens of histidine-95, the ^{15}N spectrum was measured with an enzyme sample of the H103Q·H185Q mutant enriched in ^{15}N *only* at the $N^{\delta 1}$ position of histidine-95. As expected, the ^{15}N spectrum of this singly labeled mutant shows one peak, at 121.2 ppm (spectrum not shown). Table I shows that in a neutral imidazole the high-field peak corresponds to an "α-type" nitrogen (Bachovchin, 1986), so $N^{\epsilon 2}$ carries a proton in histidine-95. The $N^{\delta 1}$ of histidine-95 at 121.3 ppm is a "β-type" nitrogen (Bachovchin, 1986) and does not carry a covalently bonded proton. This tautomeric arrangement is entirely consistent with that deduced from careful analysis of the X-ray structure of the yeast isomerase (Lolis et al., 1990).

[1] ^{15}N chemical shifts are here reported such that positive values are upfield from the 1 M HNO_3 reference.

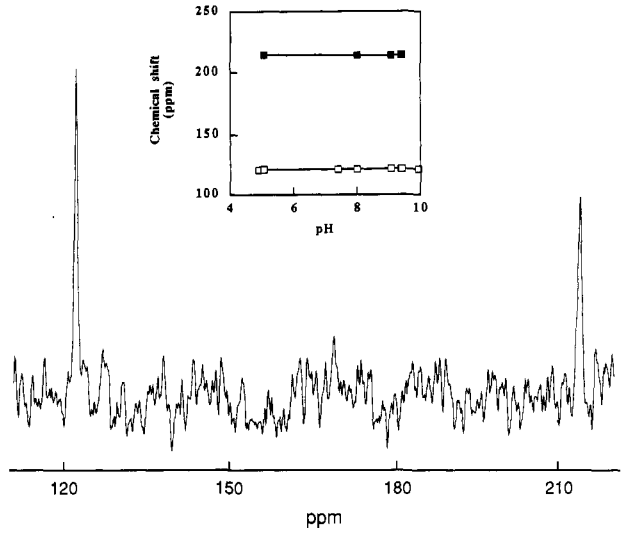

FIGURE 5: ^{15}N NMR spectrum of unliganded H103Q·H185Q triosephosphate isomerase at pH 9.4. The enzyme sample was between 2 and 3 mN in isomerase active sites. The relaxation delay was 0.9 s, the acquisition time was 0.41 s, and the decoupler frequency was 9100 Hz. This is an accumulation of about 35000 transients. Inset: Chemical shift of $N^{\epsilon 2}$ (■) and $N^{\delta 1}$ (□) of histidine-95 of the unliganded H103Q·H185Q isomerase as a function of pH. For details, see Experimental Procedures.

When the observed chemical shift values reported here are compared with the "typical" values listed in Table I, it is evident that the $N^{\delta 1}$ peak is at lower field than the value proposed for a typical β-type nitrogen. Moreover, the $N^{\epsilon 2}$ peak of histidine-95 is at a slightly higher field than that suggested for an α-type nitrogen. These differences may be due to differences between proteins, though in the case of $N^{\delta 1}$ the discrepancy is rather large, particularly when the structure of the enzyme is considered. The crystallographic data indicate that $N^{\delta 1}$ of histidine-95 is within hydrogen-bonding distance of the main-chain NH group of glutamate-97 (Lolis et al., 1990). A hydrogen-bonded β-type nitrogen is expected to have a chemical shift near 138 ppm, so that the position of this peak at 121.3 ppm suggests not only that this nitrogen is *not* the recipient of a hydrogen bond, but that it is even less shielded than a typical non-hydrogen-bonded, β-type imidazole nitrogen. The position at 213.5 ppm of the $N^{\epsilon 2}$ nitrogen of His-95 is at slightly higher field than a typical non-hydrogen-bonded α-type nitrogen, indicating that in the unliganded enzyme histidine-95 is not a hydrogen bond donor. In contrast to these results, the X-ray crystallographic data suggest that, in the absence of substrate or inhibitor, histidine-95 acts as a hydrogen bond donor to glutamate-165 and to a solvent oxygen (Lolis et al., 1990). While other discrepancies between X-ray data and solution NMR data have been reported [see, for example, the apparent discrepancy between solution and crystallographic results for α-lytic protease: Smith et al. (1989)] and may reflect real differences in hydrogen bond arrangements, the present NMR results are unambiguous (and in agreement with the X-ray structure) in indicating *a neutral imidazole ring for histidine-95.*

To approximate the effect of substrate or reaction intermediate in the active site, pH variation studies were carried out with the doubly labeled [$^{15}N^{\delta 1}$,$^{15}N^{\epsilon 2}$]histidine H103Q·H185Q isomerase in the presence of the inhibitor phosphoglycolohydroxamate. Figure 6 shows the spectrum of ^{15}N-enriched H103Q·H185Q isomerase in the presence of phosphoglycolohydroxamate at 10 mM. First, the upfield $N^{\epsilon 2}$ peak shifts about 9 ppm downfield, to 204.2 ppm, indicating that $N^{\epsilon 2}$ now acts as the donor of a hydrogen bond. This new

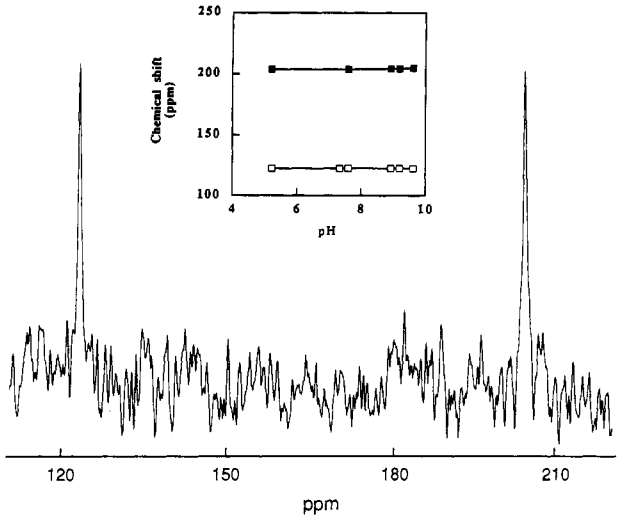

FIGURE 6: ^{15}N spectrum of histidine-95 in H103Q·H185Q triosephosphate isomerase in the presence of phosphoglycolohydroxamate (10 mM) at pH 7.6. The enzyme sample was about 3 mN in isomerase active sites. The relaxation delay was 0.65 s, the acquisition time was 0.205 s, and the decoupler frequency was 13 000 Hz. This is an accumulation of about 51 000 transients. Inset: Chemical shift of $N^{\epsilon 2}$ (■) and $N^{\delta 1}$ (□) of the H103Q·H185Q isomerase in the presence of phosphoglycolohydroxamate (10 mM) as a function of pH. For details, see Experimental Procedures.

hydrogen bond remains throughout the range of the pH titration (Figure 6, inset). In the liganded enzyme the $N^{\delta 1}$ peak also shifts slightly, to 122.6 ppm, where it remains throughout the range of pH values studied. When ^{15}N spectra were obtained in the presence of the analogue 2-phosphoglycolate, effects similar to those obtained with phosphoglycolohydroxamate were seen. The $N^{\epsilon 2}$ resonates at around 204 ppm, and $N^{\delta 1}$ at 123 ppm (data not shown). The strong hydrogen bond that is formed between $N^{\epsilon 2}$ of histidine-95 and each of these inhibitors is not unexpected if each is indeed an analogue of the enediol(ate) intermediate. In both of the isomerase mechanisms shown in Figure 2, the carbonyl oxygen of the bound substrate accepts a hydrogen bond from histidine-95. The NMR data show that this hydrogen bond is much stronger than any hydrogen bond to solvent water that may exist in the unliganded protein. The high-resolution X-ray structure of the yeast isomerase containing phosphoglycolate has recently been reported by Lolis and Petsko (1990), who have shown that phosphoglycolate binds analogously to the hydroxamate (Davenport et al., 1991).

Implications for Catalysis. The results presented above indicate that the imidazole side chain of histidine-95 is neutral in both the free and liganded forms of the isomerase and that this side chain remains neutral over the pH range of isomerase catalytic activity. The data also show that $N^{\epsilon 2}$ of histidine-95 carries a proton and acts as a hydrogen bond donor to the substrate-like inhibitors of the enzyme. In the absence of bound ligands, the chemical shift of the $N^{\epsilon 2}$ nitrogen indicates that this nitrogen is not significantly hydrogen bonded. Analogously, the chemical shift of $N^{\delta 1}$ of histidine-95 requires a tertiary nitrogen that is not the recipient of a hydrogen bond. This finding is not consistent with conclusions based on several crystal structures of triosephosphate isomerase, each of which shows that this nitrogen is positioned well (at 2.9–3.0 Å) to receive a hydrogen bond from the main-chain amide nitrogen of glutamate-97 (Lolis et al., 1990; Lolis & Petsko, 1990; Davenport et al., 1991). It seems possible that the chemical shift of this nitrogen may be affected by other features [perhaps the local α-helix (see below) or a nonaqueous microenvironment (Schuster & Roberts, 1979)] of the environment in which it finds itself.

The NMR data for the H103Q·H185Q mutant containing bound phosphoglycolohydroxamate indicate that $N^{\epsilon 2}$ of histidine-95 is a hydrogen bond donor, and we can presume from the crystal structure of the complex of the enzyme with this inhibitor that it is the carbonyl oxygen of the hydroxamate that accepts the hydrogen bond. Because the inhibitor is closely related to the substrate of the isomerase (and is believed to mimic the enediol intermediate), we can conclude that histidine-95 utilizes this hydrogen bond to polarize the substrates' carbonyl group. Such polarization has been suggested previously on the basis both of the increased chemical reactivity of bound substrate toward exogenous borohydride, and from the shift in the carbonyl group stretching frequency of bound dihydroxyacetone phosphate (Webb & Knowles, 1974; Belasco & Knowles, 1980). The existence of this polarizing hydrogen bond further suggests that histidine-95 stabilizes the enediolate intermediate, thus facilitating the enolization step of the catalyzed reaction.

Our NMR data show that the first pK_a of histidine-95 (imidazolium to imidazole) is below 4.5, which is a perturbation of at least two pK units from the value in the denatured enzyme, where preliminary results indicate a pK_a value between 6.5 and 6.7. If the second pK_a (imidazole to imidazolate) of histidine-95 has been lowered similarly [and it is known that, in substituted imidazoles, pK_a^1 and pK_a^2 vary equally together: Bruice and Schmir (1958)], then the possibility exists that the pK_a values of the catalytic imidazole and of the enediol intermediate are more nearly matched. After the enolization step (which produces the endiolate–imidazole pair), a facile proton transfer between these groups of similar pK_a would create the enediol and histidine imidazol*ate*. A second proton transfer from the other hydroxyl group of the enediol to imidazolate would then produce the other enediolate, en route to the product. As has been noted above, our NMR results rule out the possibility that mechanism A (Figure 2) describes the role of histidine-95 in the isomerase-catalyzed reaction. The data are, however, consistent with the transient formation of an enediol–imidazol*ate* pair that mediates the proton transfers between substrate oxygen atoms, as illustrated by mechanism B (Figure 2). The NMR results compel us to embrace mechanism B as the correct depiction of the role of histidine-95 in triosephosphate isomerase. To our knowledge, this is the first time that a histidine imidazolate (albeit one deriving from a side chain of lowered pK_a) has been invoked in the catalytic mechanism of an enzyme.

Implications for Structure. The resonance positions of the nitrogens in the imidazole side chain raise questions as to the nature of the environment around histidine-95. What causes the pK_a^1 of this residue to drop from about 6.5 (in the denatured enzyme) to a value below 4.5? A strong hydrogen bond to the unprotonated nitrogen $N^{\delta 1}$ would be a possible (and partial) explanation. But while the crystallographic data indicate that $N^{\delta 1}$ of histidine-95 and the main-chain nitrogen of glutamate-97 are well positioned in terms of distance and geometry to participate in a hydrogen bond, the ^{15}N NMR results presented here show that $N^{\delta 1}$ is more deshielded than a typical hydrogen bond recipient. The resonance for this nucleus is 17 ppm from the expected value for a hydrogen-bonded β-type nitrogen. While the presence of the hydrogen bond cannot be ruled out, the ^{15}N NMR data suggest that other factors deshield the nucleus and lower the pK_a of histidine-95. Similarly, $N^{\epsilon 2}$ of histidine-95 may donate a hydrogen bond to water or to glutamate-165 in the unliganded

enzyme as the X-ray data suggest, but the ^{15}N NMR data indicate an unusually shielded nucleus that becomes a hydrogen bond donor only when the enzyme is liganded to a substrate or substrate-like species.

The environment around histidine-95 immediately suggests an explanation for the perturbed pK_a that we have observed. Indeed, Hol (1985) predicted that histidine-95 of triosephosphate isomerase would have an unusually low pK_a, simply on the basis of its position at the N-terminus of a short α-helix. Empirical and theoretical data compiled by Hol (Hol et al., 1978; Hol, 1985) suggested that an amino acid that is within 3 residues of the N-terminus of an α-helix may experience a lowering of the ionization constant due to the local electrostatic effects at the helix terminus. Similarly, a residue near the C-terminus of an α-helix could have a raised pK_a due to the nearby carbonyl group dipoles. Since Hol's survey, several groups have provided elegant examples of pK_a shifts of histidine residues where the imidazole ring lies at one or other end of an α-helical segment (Šali et al., 1988; Perutz et al., 1985). Additionally, it has been shown that charged groups positioned at the termini of some α-helices can play an important role in helix stability (Shoemaker et al., 1985, 1987). There is some debate as to whether these effects come from the helix per se, or whether the helix merely provides a convenient framework for an effective alignment of the dipoles of the peptide backbone at the helix termini (Tidor & Karplus, 1991; Åqvist et al., 1991). If the helix is viewed as an array of hydrogen bond donors and acceptors, then the source of the dipole is explained by the lack of hydrogen bond donors at the C-terminus and the lack of hydrogen bond acceptors at the N-terminus. The carbonyl and amino groups that have "unsatisfied" hydrogen bonds produce a net dipole. The question of origin notwithstanding, the effects of "helix dipoles" are well established and serve to account for the unusual ionization behavior observed in the present work.

Examination of several crystal structures of triosephosphate isomerase shows a short helix from residues 95 to 102. The active site histidine-95 is positioned at the amino end of this helix (see Figure 3). The imidazole ring is "edge-on" to the helix, with $N^{\delta 1}$ close to several unsatisfied main-chain NH groups and carbonyl carbons. These NH and carbonyl carbon centers at the amino terminus of an α-helix are believed to bear most of the net positive charge that is attributable to the helical arrangement (Hol, 1985). The proximity of these positively charged elements of the α-helix accounts for the shift in pK_a and also explains the deshielding of $N^{\delta 1}$ that is seen in the ^{15}N spectrum. Indeed, it seems possible that $N^{\delta 1}$ is hydrogen bonded to the main-chain amide of glutamate-97, as suggested by the X-ray data (Lolis et al., 1990), but the shielding effect of this hydrogen bond is masked by the deshielding effect of the helix. The hydrogen bond proposed from the crystal structure may provide supplemental stabilization of the neutral imidazole or of negatively charged imidazolate. In any case, the pK_a of histidine-95 is lowered, which has a direct impact on the mechanism of catalysis by this enzyme.

In summary, we have described experiments that demonstrate that the imidazole side chain of histidine-95 in triosephosphate isomerase is neutral over the whole pH range of activity of this enzyme. Our experiments, combined with knowledge of the X-ray crystal structure of the enzyme, suggest that an α-helix aimed at the active site is responsible for the perturbed pK_a. Yet the reasons why the enzyme has evolved to use a neutral histidine as a general acid are not clear. Perhaps if histidine-95 had carried a positively charged imidazolium side chain, the basicity of glutamate-165 would have been lowered and the catalytic ability of the enzyme would have been impaired. Possibly the enzyme evolved to "match" the pK_a values of the catalytic imidazole nitrogen and the substrate's enediol oxygen in order to allow a rapid proton transfer between these sites. Maybe the use of imidazolium as a general acid would have caused the intermediate to be *too* stable, thereby slowing the reaction catalyzed by the enzyme. Indeed, Bash et al. (1991) have performed ab initio and molecular dynamics calculations that suggest that if the side chain of histidine-95 were positively charged, the imidazole–enediol pair formed during the catalyzed reaction would have a very low free energy. Such a deep thermodynamic well in the free energy profile would obviously be inconsistent with the efficient catalysis that the isomerase mediates. This study further indicated that the imidazol*ate*–enediol pair (resulting from the use of a neutral imidazole side chain of histidine-95) would have a free energy that is consistent with the known kinetic parameters for triosephosphate isomerase. Whether or not the details of this analysis will turn out to be correct, it is interesting that theory and experiment have here agreed upon a result that runs counter to the initial prejudices of mechanistic chemistry.

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Kinetic Competence of an Externally Generated Dienol Intermediate with Steroid Isomerase†

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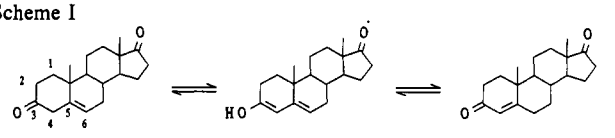
ABSTRACT: The putative intermediate dienol (**2**) in the steroid isomerase (KSI) catalyzed conversion of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) has been independently generated and tested as a substrate for KSI. At pH 7, dienol **2** is converted by KSI to a mixture of **1** (46%) and **3** (54%). The apparent second-order rate constant for reaction of **2** with KSI to produce **3** ($k_{cat}/K_m = 2.3 \times 10^8$ M^{-1} s^{-1}) is similar to that for reaction of **1** with KSI ($k_{cat}/K_m = 2.1 \times 10^8$ M^{-1} s^{-1}), demonstrating that **2** is kinetically competent. Isomerization of **1** by KSI in D_2O gives only 5% of solvent deuterium incorporated into the product **3**. When **2** reacts with KSI in D_2O, and the product **3** is isolated (from direct reaction of **2** and from subsequent conversion of the **1** initially formed), ca. 80 atom % deuterium is located at C-6β, confirming that protonation of the dienol by KSI occurs at the same face as the proton transfer in the KSI catalyzed reaction of **1** to **3**.

The 3-oxo-Δ^5-steroid isomerase (KSI)[1] from *Pseudomonas testosteroni* catalyzes the isomerization of a variety of Δ^5-3-oxosteroids to their conjugated isomers [see Pollack et al. (1989b) for a review]. This reaction serves as a prototype for a variety of enzyme-catalyzed allylic rearrangements that involve a 1,3-hydrogen shift between carbon atoms (Schwab & Henderson, 1990). KSI is an extremely efficient enzyme, with k_{cat}/K_m values approaching the diffusion-controlled limit for 5-androstene-3,17-dione and 5-pregnene-3,20-dione as substrates. Early studies (Wang et al., 1963; Malhotra & Ringold, 1965) suggested that the enzymatic isomerization

Scheme I



1 2 3

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[1] Abbreviations: KSI, 3-oxo-Δ^5-steroid isomerase; HSD, 3α-hydroxysteroid dehydrogenase; PAGE, polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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