Direct Evidence for the Exploitation of an α-Helix in the Catalytic Mechanism of Triosephosphate Isomerase†

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ABSTRACT: In previous work, we have shown that the first (and, presumably, the second) pK_a of the active-site histidine-95 in triosephosphate isomerase has been lowered by about 2 units [Lodi, P. J., & Knowles, J. R. (1991) *Biochemistry 30*, 6948–6956]. One reason for the perturbed pK_a of this residue appears to be its location at the N-terminus of a short α-helix that runs from residues 95 to 102. Fortuitously, the residue at the C-terminus of this helix is also a histidine residue (histidine-103), and the existence of a histidine side chain at *each* end has allowed us directly to implicate the helix in the perturbation of the pK_a value of histidine-95. ^{15}N NMR titration studies of the native enzyme and ^{13}C NMR titration studies of the denatured enzyme show that while the pK_a of histidine-95 is *lowered* by a least 2 units in the folded versus the unfolded state, the pK_a of histidine-103 is *raised* by about 0.6 unit on protein folding. These complementary effects on the pK_a values of histidine-95 and histidine-103 suggest that the α-helix is indeed responsible for the perturbation of the pK_a values. The larger effect on the pK_a of histidine-95 is readily rationalized in terms of the local structure of the enzyme. The disparity in the perturbation for the two histidine side chains illustrates how an α-helix can be functionally utilized by proteins, directly to affect (as in the present case) the chemistry of catalysis by an enzyme.

Triosephosphate isomerase catalyzes the interconversion of (*R*)-glyceraldehyde 3-phosphate (GAP)[1] and dihydroxyacetone phosphate (DHAP) as shown in Figure 1. The enzyme is a homodimer, with a subunit molecular weight of about 26 500 (Putman et al., 1972). Dihydroxyacetone phosphate binds at the active site, and enolization occurs by the abstraction of the *pro-R* hydrogen from C-1 by glutamate-165 (Waley et al., 1970; Hartman, 1971; de la Mare et al., 1972; Banner et al., 1975; Lolis et al., 1990). This process thus generates an enediolate that may be protonated on O-2 by a general acid catalyst on the enzyme to produce an intermediate enediol (Bloom & Topper, 1956; Rieder & Rose, 1959). In order for this intermediate to collapse to product GAP, the abstraction of the O-1 proton by an enzymic base then creates the *other* enediolate. As the carbonyl group of GAP is formed, carbon protonation occurs at C-2, and GAP is then released from the enzyme. There is good evidence that a single enzymic functionality, histidine-95, is responsible for mediating the proton transfers to and from the substrate's oxygen atoms (Nickbarg et al., 1988; Komives et al., 1991; Lodi & Knowles, 1991).

The crystal structure of yeast triosephosphate isomerase shows that there is a short α-helix between residues 95 and 102 (Lolis et al., 1990). The presence of the catalytic acid histidine-95 at the N-terminus of this helix invites the view that the "helix dipole" (Wada, 1976; Hol et al., 1978; Hol, 1985) may lower the first (and, presumably, the second) pK_a of the imidazole side chain. Experiments reported previously (Lodi & Knowles, 1991) have shown that the pK_a of histidine-95 is indeed unusually low in the folded enzyme, and this finding led to the suggestion that the neutral imidazole side chain of histidine-95 acts as an acid, itself becoming transiently negatively charged during the catalytic interconversion. Such an interesting (and unprecedented) mechanistic possibility adds significance to efforts to understand *how* the enzyme lowers the pK_a of histidine-95. Aiding these efforts, the wild-type enzyme from yeast has a second histidine, near the C-terminus of the same helix, at position 103. The existence of this residue provides an unusual opportunity to investigate the complementary effects of the helix dipole at each of the helix termini.

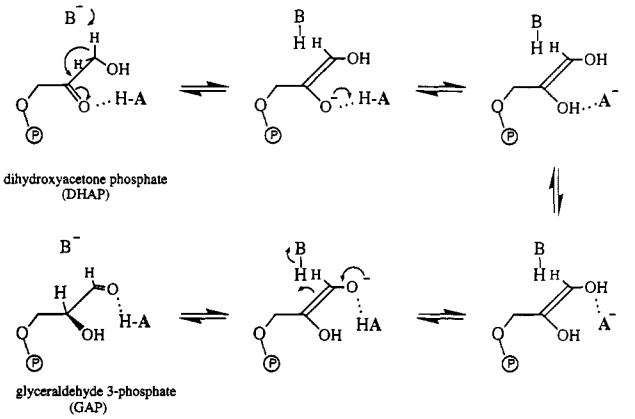

FIGURE 1: Mechanistic pathway for the TIM-mediated interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. B$^-$ is the catalytic base (glutamate-165), and H-A is the catalytic acid (histidine-95).

There are two views about how an α-helix interacts with surrounding groups. From one perspective, the "helix dipole effect" is considered to be a directional phenomenon caused by the small dipoles of each of the peptide units that are arrayed in fixed positions at the termini of the helix. These units are available for, and well-positioned to participate in, hydrogen bonding, so that a nearby molecule (or nearby parts of the same molecule) may take advantage of the accessible,

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[1] Abbreviations: GAP, (*R*)-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; TIM, triosephosphate isomerase.

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unsatisfied hydrogen-bonding functionalities at the helix termini. In this model, an interacting group must be near to, *and* able to form hydrogen bonds with, the peptide backbone at the N- or C-terminus, and the "helix dipole effect" is simply the result of that well-positioned interaction (Aqvist et al., 1991). Alternatively, the helix dipole can be viewed as a purely electrostatic effect, whereby a partial net charge exists at the helix termini and nearby groups are perturbed by this field. In this view, it is not necessary for a hydrogen bond actually to be shared with the α-helix (although the presence of a hydrogen bond may contribute to the effect of the helix dipole) because the dipole can be treated as a partial charge centered at each helix terminus (Nicholson et al., 1988, 1991). Regardless of the precise nature of the α-helix dipole, however, several laboratories have gathered data to substantiate the idea that these partial charges can be exploited to increase protein stability (Shoemaker et al., 1985, 1987; Šali et al., 1988; Serrano & Fersht, 1989; Nicholson et al., 1988, 1991; Sancho et al., 1992), to aid in the binding of small molecules (Pflugrath & Quiocho, 1988; Jacobson & Quiocho, 1988), and to improve catalytic efficiency (Lodi & Knowles, 1991). In addition, the electric field at the N-terminus of several short helical peptides has recently been measured by exploiting the internal Stark effect of a probe molecule attached to the helices through a peptide linkage (Lockhart & Kim, 1992).

Here we describe experiments that implicate the active-site helix as an important contributor to the perturbed pK_a of histidine-95, and thus show that the helix plays a direct role in catalysis by triosephosphate isomerase.

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). Oligo-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).

Reagents for cell growth, and for purification and assay of the enzyme, were as described by Komives et al. (1991). Enzyme-grade urea was obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Methods. The gene encoding triosephosphate isomerase from baker's yeast was obtained from E. Lolis as an insert into M13mp18 phage. An *Nco*I site was introduced at the 5′ end of the gene, and a *Pst*I site was introduced at the 3′ end of the gene as described previously (Lodi & Knowles, 1991). All mutagenesis procedures followed the methods of Eckstein (Nakamaye & Eckstein, 1986). The wild-type isomerase gene was subcloned into a modified pKK223-3 vector described previously (Lodi & Knowles, 1991). Some experiments were carried out with the H103Q mutant enzyme. To change histidine-103 to glutamine, the mutagenic primer 5′-CATCTTCTTGGAAGTAAG-3′ was used, and the mutagenesis procedure was carried out while the gene remained in M13mp18. The mutant isomerase was then subcloned into the modified pKK223-3 vector. The entire isomerase gene was then sequenced to confirm that the only mutation encoded the H103Q change.

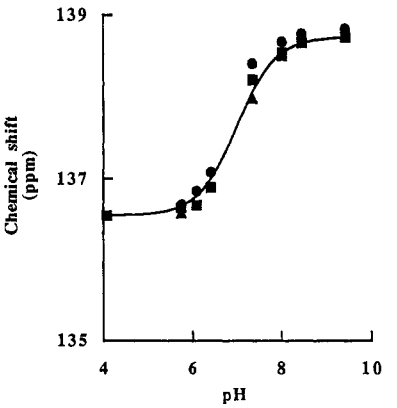

FIGURE 2: ^{13}C NMR chemical shift versus pH of ^{13}C-enriched wild-type triosephosphate isomerase in the presence of 8 M urea. Titration curves (uncorrected for the presence of urea) are of histidine-95 (●), histidine-103, and histidine-185 (■, ▲, unassigned) in the denatured enzyme. The curve is theoretical for a pK_a of 6.99. After correction for the presence of urea, the observed pK_a values are 6.43, 6.58, and 6.68.

[^{13}C]Histidine Incorporation. The overproduction and purification of wild-type and mutant yeast isomerases enriched with [$^{13}C^{\epsilon 1}$]histidine have been described previously (Lodi & Knowles, 1991).

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were recorded at 125.8 MHz on a Bruker AM500 spectrometer, with a 5-mm standard probe equipped to detect ^1H, ^{13}C, ^{15}N, and ^{31}P.

Enzyme Samples. For the experiments using native isomerase, the ^{13}C NMR samples were 0.6–3 mN in enzyme active sites. [Since the enzyme is a homodimer, millimolar is used to signify the concentration of dimers, and millinormal is used to signify the concentration of enzyme protomers.] The enzyme samples were dissolved in 100 mM Tris-HCl buffer and were prepared by concentration of a dilute solution of enzyme in a Centricon filtration unit. After concentration, the NMR samples were brought to 10% D_2O (v/v). The pH of a sample was changed by diluting it into 100 mM Tris-HCl buffer that had been adjusted to the desired pH, and then concentrating the sample. Occasionally, 0.1 or 1 M HCl was added to a dilute enzyme solution before concentration in order to adjust the pH. The pH of the sample was measured with a glass combination electrode, and values reported are the meter readings, uncorrected 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 this standard (De Marco, 1977). Spectra were acquired at room temperature by using a 30° radio-frequency pulse with a spectral width of about 30 000 Hz and 16K data points. The decoupler frequency was set to 9100 Hz, and a relaxation delay of 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 changed by more than 0.05 pH unit during the run.

For the experiments involving denatured wild-type isomerase, the ^{13}C NMR samples were 0.4 mN in enzyme active sites and contained urea (8 M), D_2O (10% v/v), and (trimethylsilyl)propionic acid (2 mg/mL). The pH of the sample was changed by adding a very small amount of 1 or 10 M HCl or 1 M NaOH, or, if a large pH change was desired, by adding a solution containing 8 M urea and either HCl or NaOH. Spectra were acquired at room temperature as described above, except that the decoupler frequency was set to either 9100 or 11 000 Hz. The pH of the sample was measured before and after spectral data accumulation, and with four of the eight pH values represented on the graph in Figure 2, the pH of the

sample changed during accumulation by 0.02 pH unit or less. However, during three of the reported accumulations, the pH changed by 0.1–0.2 pH unit, and during one of the accumulations, the pH value changed by 0.22 pH unit.

For the experiments involving denatured mutant isomerase (either H103Q or H103Q·H185Q), the ^{13}C NMR samples were 0.8–1.2 mN in enzyme active sites, and contained urea (8 M, added as a solid), Tris-HCl buffer (100 mM), D_2O (10% v/v), and (trimethylsilyl)propionic acid (4 mg/mL). The pH of a sample was changed by adding a solution of 0.5–1 M NaOH containing urea (8 M) and D_2O (10% v/v), or by adding a solution of 0.5–1 M HCl containing urea (8 M) and D_2O (10% v/v). Spectra were acquired at room temperature as described above, with a decoupler frequency of 9100 Hz. No sample suffered a pH change of more than 0.03 pH unit during data accumulation.

Histidine Samples. In the experiments to determine the pK_a of histidine in the absence or presence of urea (8 M), the NMR samples were 25 mM (*R,S*)-[$^{13}C^{\epsilon 1}$]histidine, containing D_2O (10% v/v) and (trimethylsilyl)propionic acid (4 mg/mL). Spectra were acquired at room temperature by using a 30° radio-frequency pulse with a spectral width of about 30 000 Hz and 32K data points. The decoupler frequency was 9100 Hz, and a relaxation delay of 0.1 s was used. Data were not used if the pH of the sample changed by more than 0.05 pH unit during accumulation.

Curve Fitting. The data were fit to a pH titration curve using the program KaleidaGraph, from Synergy Software (Reading, PA).

RESULTS

In earlier work using ^{15}N NMR, the pK_a values of histidine-95 and histidine-103 in the native yeast triosephosphate isomerase have been shown to be lower than 4.5 and 7.46, respectively (Lodi & Knowles, 1991). However, the titration curve for histidine-103 was determined in 95% (v/v) D_2O, and to ensure comparability with later experiments, the pK_a value was redetermined by ^{13}C NMR (using isomerase enriched at the $C^{\epsilon 1}$ position of the histidine residues) in 10% (v/v) D_2O. This experiment gave a pK_a for histidine-103 of 7.29 under these conditions (data not shown). All subsequent experiments were carried out in 10% (v/v) D_2O.

When the enzyme is in the presence of 5 M urea, the ^{13}C NMR spectrum changes markedly. The broad signals that were observed for the folded isomerase are replaced by very sharp signals, indicating that the protein is denatured (data not shown). The experiments on the denatured enzyme that we describe here were carried out in 8 M urea, under which conditions we presume that the isomerase is completely unfolded. High concentrations of urea can perturb the pK_a of the histidyl residues in proteins (Šali et al., 1988), and experiments were therefore carried out to determine the effect of 8 M urea on histidine pK_a values. The NMR spectrum of aqueous [$^{13}C^{\epsilon 1}$]histidine was measured over a range of pH values, and the pK_a of the imidazole ring was found to be 6.05 (data not shown). In 8 M aqueous urea, the pK_a of the imidazole ring was found to be 6.46 (data not shown). The difference between these two values, 0.41, has been used to correct for the presence of the denaturant.

The NMR spectrum of a solution of triosephosphate isomerase (labeled with ^{13}C at the $C^{\epsilon 1}$ position of histidine residues) in 8 M urea was measured between pH 4.08 and pH 9.42. The resulting titration curve is shown in Figure 2. All three histidines in the denatured wild-type isomerase have similar pK_a values: 6.43, 6.58, and 6.68 (after correcting for the presence of urea). Although the enzyme is believed to be fully denatured, the pK_a values for the histidine side chains are not identical, presumably because of the slightly differing environments of these residues along the unfolded polypeptide chain.

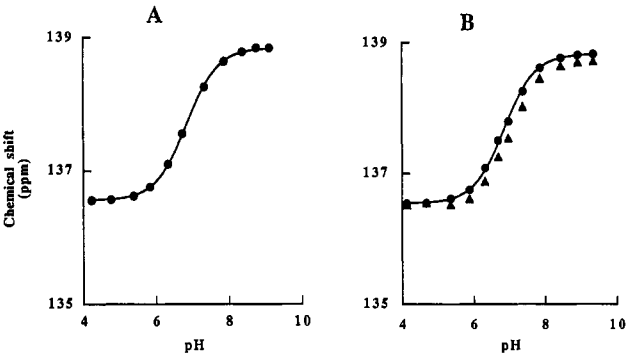

FIGURE 3: ^{13}C NMR chemical shift versus pH of ^{13}C-enriched mutant isomerases in the presence of 8 M urea. (A) Titration curve of histidine-95 for denatured H103Q·H185Q mutant isomerase (uncorrected for urea). The curve is theoretical for pK_a 6.85; after correction for urea, the pK_a is 6.44. (B) Titration curves of histidine-95 (●) and histidine-185 (▲) for denatured H103Q mutant isomerase (uncorrected for the presence of urea). The curve is theoretical for pK_a 6.86; after correction for urea, the pK_a values are 6.46 and 6.62.

To determine which of the three pK_a values corresponds to histidine-95, a sample of the double mutant isomerase H103Q·H185Q enriched with ^{13}C at the $C^{\epsilon 1}$ position of the only remaining histidine (histidine-95) was examined in 8 M aqueous urea. The ^{13}C NMR spectrum showed a single peak as expected. The chemical shift of this peak plotted as a function of pH is shown in Figure 3A, and the pK_a (corrected for the presence of 8 M urea) is 6.44. This value is experimentally indistinguishable from the value of 6.43 obtained for the denatured wild-type isomerase, and so the pK_a of histidine-103 in the denatured isomerase is therefore either 6.58 or 6.68.

The assignment of the pK_a values of histidine-103 and histidine-185 in the denatured isomerase was attempted by carrying out a pH titration using purified H103Q isomerase. The H103Q mutant contains two histidines, at positions 95 and 185. Figure 3B shows the plot of chemical shift against pH from 4.14 to 9.33 for the ^{13}C-labeled denatured H103Q mutant. When corrected for the effect of 8 M urea, one histidine titrates with a pK_a of 6.46 and is assigned to histidine-95. The other histidine in the mutant is histidine-185, which titrates with a pK_a of 6.62. This pK_a value falls between the values of 6.58 and 6.68 found in the wild-type titration. For present purposes, therefore, we must be satisfied with the knowledge that the pK_a of histidine-103 in the denatured isomerase is 6.58 or 6.68.

DISCUSSION

The imidazole ring of the active-site histidine-95 of triosephosphate isomerase has a first pK_a that is below 4.5 (Lodi & Knowles, 1991), a value that is more than 2 pK_a units lower than is typical for a histidine in a random polypeptide chain (Richarz & Wüthrich, 1978). One possible cause of this anomalously low pK_a may be a shared hydrogen bond between the main chain amide of glutamate-97 and $N^{\delta 1}$ of histidine-95. If $N^{\delta 1}$ is the recipient of a hydrogen bond, it cannot itself become protonated without severing the hydrogen bond, so the pK_a of histidine-95 would be lowered by this interaction. In addition to this hydrogen bond, however, the imidazole group lies at the end of a short α-helix (from residues

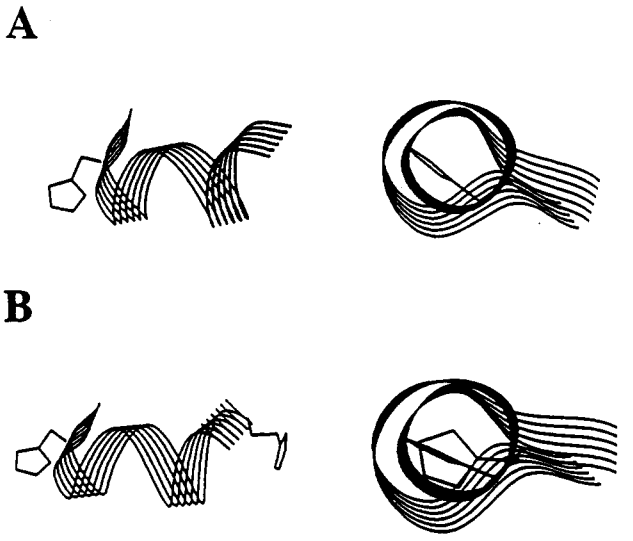

FIGURE 4: α-Helix from residues 95 and 102 in triosephosphate isomerase. (A) Two views, showing the position of histidine-95 with respect to the helix. (B) Two views, showing both histidine-95 and histidine-103. Histidine-95 is "edge-on" to the helix, and histidine-103 acts like a "cap" on the helix [coordinates from Davenport et al. (1991)].

95 to 102) in a position that is revealed in each of the several known X-ray crystal structures of the isomerase (Alber et al., 1981; Lolis et al., 1990; Davenport et al., 1991). In an extensive review, Hol has examined the crystal structures of many proteins and has predicted that several enzymes, including triosephosphate isomerase, contain helices aimed at the active site that may affect catalysis by altering the pK_a of an active-site residue (Hol, 1985).

Histidine-95 is located at the N-terminus of the two-turn active-site helix, and the imidazole ring is centered and edge-on to the helix axis (Figure 4A). The partial positive charge associated with the N-terminus of the α-helix is expected to lower the pK_a of the imidazolium ring. Indeed, the hydrogen bond to $N^{\delta 1}$ and the α-helix are likely to be interrelated, and both may contribute to the lowering of the pK_a of histidine-95: one way to view an α-helix is that it is a structural unit that can cause favorable alignment of a hydrogen bond donor at its N-terminus (Aqvist et al., 1991). Another viewpoint holds that while a hydrogen bond is not required for a helix to perturb the pK_a of a terminal residue, its presence can increase the effect on that residue (Nicholson et al., 1988, 1991). The well-aimed helix and the hydrogen bond from a residue at the N-terminal of the helix probably both contribute to the "helix dipole effect" by which the pK_a of histidine-95 is lowered.

At this point, we can only speculate as to why the pK_a of histidine-95 has been perturbed by the enzyme. Perhaps a positively charged imidazolium would upset a delicate charge balance in the active site, decreasing the catalytic potency of the enzyme. It is also possible that the enzyme has evolved to match the pK_a of the catalytic acid (histidine-95) with the pK_a of the intermediate enediol to allow a more rapid proton transfer between them. Possibly, proton transfer from a histidine imidazolium to an enediolate to create an imidazole·enediol pair would overstabilize the intermediate of the reaction, as has been suggested by ab initio and molecular dynamics calculations (Bash et al., 1991). A deep thermodynamic well in the free energy profile would obviously be inconsistent with the efficient catalysis that the isomerase mediates. Although the question of why the pK_a of histidine-95 has been lowered is still a topic for speculation, this paper explores *how* the structure of the enzyme produces that pK_a value.

Table I: Comparison of pK_a Values of Histidine-95 and Histidine-103 in Folded and Unfolded Triosephosphate Isomerase

	pK_a in folded isomerase	pK_a in denatured isomerase[a]	ΔpK_a
histidine-95	<4.5	6.44	−2
histidine-103	7.29	6.7	+0.6

[a] pK_a values are corrected for the presence of 8 M urea (see the text).

We begin with the premise that the enzyme may use the dipole associated with the α-helix to help lower the pK_a of histidine-95. Indeed, 5 of the 8 residues in this helix are conserved in all 13 triosephosphate isomerases for which sequence data have been reported (Wierenga et al., 1992), suggesting that the helix is important to the action of the enzyme. Although the position of the histidine-95 side chain with respect to the helix suggested that a helix dipole could perturb the pK_a of the imidazole and thereby affect the mechanism of catalysis (Hol, 1985; Lodi & Knowles, 1991), no direct evidence existed to support this assumption. To test the existence and effect of a helix dipole in triosephosphate isomerase, we examined the amino acids at each end of the helix. It is clear that histidine-95, at the N-terminus of the helix, has a pK_a that is lowered from its normal value. If the residue at the C-terminus of the helix could be shown to have a pK_a that is *raised* above its unperturbed value, then the helix would be implicated as a contributor to both of these complementary effects. In the wild-type yeast isomerase, the residue at the C-terminus of the helix in question happens also to be a histidine (histidine-103), which is a surface residue that appears not to interact with any charged groups on the enzyme (Lolis et al., 1990). Experiments were therefore carried out to ascertain whether the helix has complementary effects on histidine-95 and histidine-103.

In principle, the effect that the isomerase secondary structure has on the pK_a of a particular residue can be determined by comparing the pK_a of the specified residue in the folded and in the unfolded enzyme. Since there are other features of enzymes besides helix dipole effects that may affect the pK_a of an ionizable residue, including hydrogen bonds and the presence of nearby charged or aromatic residues, the measured difference in the pK_a values in the folded and unfolded states reflects the sum of all of the effects that the structure imposes on the residue. Careful scrutiny of the three-dimensional structure of the enzyme is therefore necessary to implicate a single motif as the cause for a perturbed pK_a. While it is impossible to be certain that no structural element other than the α-helix is responsible for altering the pK_a values of histidine-95 and histidine-103, X-ray crystal structures show that the major interactions of histidine-95 are with amino acid residues that are part of its associated helix. Furthermore, except for its proximity to the helix, histidine-103 appears not to interact with any charged or aromatic residues of the protein (Lolis et al., 1990).

In Table I, the pK_a values of histidine-95 and histidine-103 in the folded and in the denatured isomerase are compared. While the pK_a of histidine-95 is lowered by at least 2 units in the folded enzyme, the pK_a of histidine-103 is *raised* by between 0.6 and 0.7 unit. These data indicate that some element of the folded enzyme perturbs the pK_a values of these two histidines in opposite directions. The helix dipole associated with the active-site α-helix may well provide the explanation of these complementary effects. By virtue of its

position at the N-terminus of the α-helix, the pK_a of histidine-95 is expected to be lower in the folded enzyme compared to the denatured enzyme, and this is what is observed. Correspondingly, the location of histidine-103 at the C-terminus of the α-helix should result in a higher pK_a for this residue in the folded than in the denatured enzyme, and the pK_a of this residue is raised, as expected.

The interaction energy between histidine residues and the α-helix can be calculated using eq 1 or 2. $\Delta G_{aa\text{-}N}$ is the contribution to the free energy of stabilization of the protein by the interaction between an α-helix and an amino acid at its N-terminus, $\Delta G_{aa\text{-}C}$ is the contribution to the free energy of stabilization of the protein by the interaction between an α-helix and an amino acid at its C-terminus, pK_a^F is the pK_a of the ionizing amino acid when the enzyme is in the folded state, and pK_a^U is the pK_a of the amino acid when the enzyme is unfolded (Cantor & Schimmel, 1980; Nicholson et al., 1991).

$$\Delta G_{aa\text{-}N} = 2.303RT(pK_a^F - pK_a^U) \quad (1)$$

$$\Delta G_{aa\text{-}C} = 2.303RT(pK_a^U - pK_a^F) \quad (2)$$

These equations show that the pK_a of a histidine in the folded isomerase and in the denatured isomerase can be used to obtain the energy of the interaction between the histidine and the folded enzyme. The enzyme may provide several sources of interaction with histidine-95, but the crystal structures indicate that the predominant interactions are with the α-helix and the residues that comprise it (Davenport et al., 1991). Histidine-95 shows a difference in pK_a that corresponds to a stabilizing interaction of at least 2.7 kcal/mol, and histidine-103 exhibits a pK_a difference corresponding to a net interaction energy of about 0.8 kcal/mol. In a recent paper, Nicholson et al. (1991) used site-directed mutagenesis to determine the effect of charged residues at the termini of the helices of T4 lysozyme. The interaction energies between the various residues and the termini of the helices were found to lie between 0.6 and 2.1 kcal/mol. In earlier work, Šali et al. (1988) calculated a favorable interaction energy of 2.1 kcal/mol for an α-helix in barnase and the histidine at its C-terminus. While the energy calculated in the present work for histidine-103 of the isomerase is similar to some of the lower values found for lysozyme (Nicholson et al., 1991), the interaction energy found for histidine-95 is greater than either of the examples cited. Recently, Lockhart and Kim (1992) have estimated the electric field at the N-terminus of several synthetic helices by measuring the internal Stark effect of a neutral probe molecule [4-(methylamino)benzoic acid] attached to the helix by a peptide linkage. The carbonyl group of the probe receives a hydrogen bond from an N-terminal amide nitrogen of the helix. It was found that the absorbance maximum of the probe shifts upon being placed at the N-terminus of a helix, and corresponds to a change in the transition energy of 1.6 kcal/mol. The shift was less under conditions that disturbed the structure of the helix, and at high ionic strength (Lockhart & Kim, 1992). These results provide further evidence on how an α-helix can affect a neighboring group at the helix terminus. As for the present work, although one can only speculate on the reasons why an exceptionally low pK_a of histidine-95 may be useful in catalysis, it is evidently no accident that triosephosphate isomerase has evolved with a neutral histidine residue in the active site.

There are several possible explanations for the difference in the effect that the folded enzyme has on the pK_a of histidine-95 and histidine-103. First, while histidine-95 is located in the relatively buried active site, histidine-103 is on the surface of the enzyme and appears to be freely accessible to solvent water (Lolis et al., 1990; Davenport et al., 1991). If the solvating water partially negates the charge from the dipole by satisfying the hydrogen-bonding capability of the residues at the terminus, then the effect of the helix dipole on histidine-103 will be lower. Second, histidine-103 and histidine-95 are oriented differently with respect to the α-helix. The imidazole ring of histidine-103 acts as a "cap" to the helix, whereas the ring of histidine-95 is "edge-on" (see Figure 4B; Davenport et al., 1991). Third, the relative sharpness of the ^{13}C resonance of histidine-103 in comparison with the other two histidines in the enzyme (data not shown) indicates that this side chain has more freedom to rotate than the other two, possibly because histidine-103 is not constrained by intramolecular hydrogen bonds. The crystal structures of the isomerase support the idea that histidine-103 is not rigidly held in one position; the atomic temperature factors are high for the atoms in this residue, and the position of $N^{\delta 1}$ in histidine-103 with respect to nearby atoms in one subunit can be quite different (by as much as 2.1 Å) from its position in the other subunit (Davenport et al., 1991). Finally, the difference in the perturbation of the pK_a values of histidine-95 and histidine-103 may derive from the existence of a strong hydrogen bond between $N^{\delta 1}$ of histidine-95 and the main chain amide of glutamate-97 at the N-terminus of the α-helix. Histidine-103 appears not to be involved in hydrogen bonding with an "unsatisfied" carbonyl oxygen of the helix.

It is noteworthy that our study of the active-site helix of triosephosphate isomerase may reconcile the two competing viewpoints regarding the effect of the "helix dipole". While some evidence has suggested that the dipole can be treated as an electric field that affects nearby residues even in the absence of any shared hydrogen bonds (Nicholson et al., 1991), other analyses indicate that the dipole effect can only be "harnessed" by hydrogen bonding with the unsatisfied main chain peptide links at the helix termini (Aqvist et al., 1991). Since histidine-103 appears to form no hydrogen bonds with the helix that it terminates, it may correspond to the first case. In contrast, histidine-95 shares a hydrogen bond with the main chain amide of glutamate-97, and seems to be an example supporting the latter view. Although we have only studied one enzyme, a reasonable conclusion is that the "helix dipole effect" has two components. The major component arises from direct participation in a hydrogen bond with one of the well-positioned dipolar main chain peptide units. A lesser effect is manifest as the electric field deriving from the helix, that perturbs the ionization constant of nearby residues but does not require an explicit hydrogen-bonding interaction.

In this report, we present evidence that an active-site α-helix may be responsible for the perturbed pK_a of histidine-95 in triosephosphate isomerase. Histidine-95 is presumed to act as a general acid in the isomerase, so the α-helix apparently participates directly to affect the catalytic mechanism of the enzyme. To our knowledge, this is the first evidence of such participation by an α-helix.

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