Triosephosphate Isomerase Requires a Positively Charged Active Site: The Role of Lysine-12†

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ABSTRACT: The role of lysine-12 at the active site of yeast triosephosphate isomerase has been elucidated by a combination of site-directed mutagenesis, Fourier transform infrared spectroscopy, enzyme kinetics, and X-ray crystallography. Several lines of evidence suggest that the mutant isomerase in which lysine has been changed to methionine cannot bind substrate. This mutant enzyme has no detectable catalytic activity, and infrared experiments show no evidence of binding dihydroxyacetone phosphate nor dihydroxyacetone sulfate to the active site. Furthermore, crystals of the enzyme grown in the presence of phosphoglycolohydroxamate, a potent reaction intermediate analog, show an open active site with no inhibitor bound. Mutation of lysine-12 to arginine produces a protein with a value K_m elevated by a factor of 22, a V_{max} reduced by a factor of 180, and a K_i for phosphoglycolohydroxamate elevated by a factor of 290. Mutation of lysine-12 to histidine produces an enzyme that shows virtually no catalytic activity at neutral pH, but below pH 6.1 this enzyme is active, suggesting that protonation of the histidine in this mutant is required for activity. These studies, together with the structural results reported in an accompanying paper, provide convincing evidence that a positive charge is required for substrate binding at the active site of triosephosphate isomerase and that lysine-12 provides this positive charge.

All of the 13 known amino acid sequences of triosephosphate isomerase (TIM) contain an active-site lysine at position 12 (Wierenga et al., 1992), and X-ray crystallographic studies have precisely determined the position of this residue in several different structures of the isomerase, including the enzymes from yeast (Lolis et al., 1990; Lolis & Petsko, 1990; Davenport et al., 1991), chicken muscle (Banner et al., 1975; Alber et al., 1981), and *Trypanosoma brucei* (Wierenga et al., 1991; Noble et al., 1991). In the isomerase from yeast, the crystal structure clearly shows that the side chain interacts with both the substrate and other amino acid residues in the protein. The function of this residue in the catalytic mechanism of TIM has largely remained obscure, except that it appears not to be involved directly in polarization of the substrate carbonyl groups, a role that seems to be fulfilled by the active-site histidine-95 (Komives et al., 1991).

X-ray crystallographic studies on the yeast isomerase complexed with the reaction intermediate analog phosphoglycolohydroxamate (PGH) are believed to give an indication of the structure of the enzyme as it carries out the catalytic reaction. In these structures (Figure 1), the ϵ-amino group of lysine-12 is 2.7 Å from the O2 of the substrate (the oxygen that would occupy the carbonyl position in dihydroxyacetone phosphate), 5 Å from the O1 of the substrate (the oxygen that would occupy the carbonyl position in glyceraldehyde 3-phosphate), 3.4 Å from the bridging oxygen of the phospho group, and 4.4 Å from the other peripheral oxygens of the phospho group. These distances suggest that the lysine may interact with the substrate to neutralize some of the negative charge of the phospho group or that it may assist in the protonation of the carbonyl groups of the substrate during catalysis.

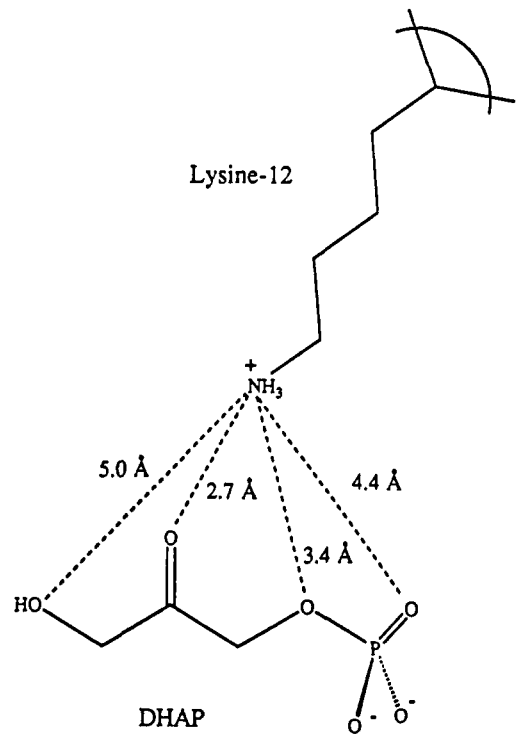

FIGURE 1: Diagram showing some of the distances between atoms of the substrate, DHAP, and the amino group of lysine-12. These distances are measured from the crystal structure of the yeast wild-type TIM–PGH complex from isosteric atoms in PGH to the lysine amino group (Davenport et al., 1991).

The possibility that lysine-12 protonates the substrate's carbonyl oxygen has effectively been ruled out by experiments in which the stretching frequencies of the substrate carbonyl

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groups have been measured by using Fourier transform infrared spectroscopy (FTIR). These experiments first indicated that the carbonyl group of dihydroxyacetone phosphate was polarized on binding to the active site of the enzyme. The stretching frequency of this group shifts 19 cm^{-1} to lower frequency compared with that of dihydroxyacetone phosphate free in solution (Belasco & Knowles, 1980). Later experiments in which similar measurements were made on substrate bound to a mutant TIM in which histidine-95 was changed to a glutamine (H95Q) showed a complete loss of substrate carbonyl polarization (Komives et al., 1991). It should be noted that bound substrate can be easily distinguished from free substrate because substrate that is bound has a much narrower bandwidth than free substrate, presumably due to the decreased conformational flexibility of the bound material. It was thus clear from the FTIR experiments on the H95Q mutant that the substrate was bound at the active site, but that its carbonyl groups were not polarized by the enzyme. Since lysine-12 was still present in the H95Q mutant, but the substrate carbonyl groups were not polarized, we inferred that lysine-12 does not contribute to the polarization of the substrate carbonyl groups. The X-ray crystal structure of the H95Q mutant confirmed this view since the positions of the substrate analog and lysine-12 are the same as those in the wild-type enzyme. Thus, the lack of substrate carbonyl group polarization in this mutant cannot be attributed to reorganization of the active site. Since polarization by a lysine would have involved hydrogen-bond formation, we inferred that the lysine residue does not transfer a proton to the substrate carbonyl group during catalysis. These experiments suggested that if lysine-12 is involved in the catalytic mechanism of triosephosphate isomerase, its role is probably to neutralize the phosphate negative charge.

To elucidate the role of lysine-12 in the catalytic mechanism of TIM, we have replaced this residue with methionine, arginine, and histidine. These mutant proteins have been characterized extensively. The mutant in which lysine-12 is replaced with methionine (K12M) has been studied by FTIR spectroscopy and X-ray crystallography, and the structural consequences of this mutation are discussed in more detail in an accompanying paper. The kinetic constants, k_{cat} and K_m, as well as the K_i for the intermediate analog PGH have been determined for each mutant protein that showed measurable activity. The catalytic activity of the K12H mutant has also been determined as a function of pH. Together, these studies provide convincing evidence that a positive charge normally provided by lysine-12 is required for substrate binding, and therefore catalysis, by triosephosphate isomerase.

MATERIALS AND METHODS

Reagents. Dihydroxyacetone phosphate, DL-glyceraldehyde 3-phosphate diethyl acetal, glucuronolactone, histidine, streptomycin, ampicillin, reduced nicotinamide adenine dinucleotide (NADH), (4-morpholino)ethanesulfonic acid (MES), ethylenediaminetetraacetic acid disodium salt (EDTA), DEAE Sephadex A-25, Dowex-50W (H^+ form), and QAE Sephadex A-120 were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) 4000 was obtained from U.S. Biochemicals (Cleveland, OH). Deuterium oxide (99.9% labeled) was purchased from MSD Isotopes (Montreal, Quebec, Canada). DL-Glyceraldehyde 3-phosphate diethyl acetal was deprotected to DL-glyceraldehyde 3-phosphate according to the manufacturer's instructions, but using one-tenth the volume of H_2O, so that the resulting solution was 10 times more concentrated. Oligonucleotides were prepared on a Milligen 7500 DNA synthesizer according to the manufacturer's protocols and were not further purified before use. Phosphoglycolohydroxamate was a gift from J. Belasco.

Dihydroxyacetone sulfate (DHAS) was prepared according to the method of J. Belasco: Sulfur trioxide:triethylamine (0.556 g, 4.0 mmol) (Aldrich, Milwaukee WI) was mixed with dihydroxyacetone (1.2 g, 13.3 mmol) in freshly distilled dimethylformamide (60 mL) under Ar in a dry flask at room temperature. The mixture was stirred overnight and was then assayed for the presence of DHAS by using glycerol-3-phosphate dehydrogenase and NADH. The assay showed the production of 5.1 mmol of DHAS. A portion (25 mL) of product was diluted to 75 mL with water and purified on a column (4.9 × 21 cm, 100 mL) of DEAE–cellulose equilibrated with 5 mM sodium benzoate buffer (pH 4.7). The purified DHAS was eluted from the column with a linear gradient of sodium benzoate (400 mL + 400 mL, 5–100 mM). The glycerol-3-phosphate dehydrogenase assay was used to locate the fractions containing purified DHAS.

Bromohydroxyacetone phosphate (BHAP) was prepared as described by de la Mare et al. (1972). Glycerol-3-phosphate dehydrogenase was obtained from Boehringer-Mannheim and was made free of TIM activity by treatment with a 100-fold molar excess of BHAP for 1 h on ice and concentrated by ultrafiltration with three buffer changes against 100 mM triethanolamine hydrochloride (pH 7.6) containing EDTA (1 mM) to remove the excess BHAP. All other reagents were from commercial sources and were used without further purification.

Proteins. The gene for wild-type TIM from yeast was subcloned into a derivative of pBS+/− that has been described previously (Blacklow & Knowles, 1990). This phagemid vector allowed the efficient production of single-stranded DNA for mutagenesis. The phagemid contains, on an *Eco*RI–*Pst*I fragment, the *trc* promoter upstream from the complete gene for yeast TIM. The mutant in which lysine-12 was changed to methionine was constructed by using the oligonucleotide-directed mutagenesis kit available from Amersham (Arlington Heights, IL), which follows the method of Nakamaye and Eckstein (1986). The sequence of the primer used in the mutagenesis reaction was 5′-GGA AGC GTT TAA CAT AAA GTT ACC ACC-3′, which hybridizes to the coding strand of the yeast TIM gene and results in the protein sequence, GGNFMLNAS. This oligo changed the lysine at position 12 to methionine, but also inadvertently changed the glycine at position 15 to alanine. The mutagenesis was subsequently repeated to produce the single K12M mutant protein. The protein in every respect behaved identically to the double mutant K12M·G15A. The crystal structure reported in an accompanying paper is that of the double mutant K12M·G15A. The gene for the mutant protein was subsequently subcloned into the high expression vector pKK223-3 (Pharmacia LKB, Piscataway, NJ), using the unique *Eco*RI and *Pst*I sites to avoid instabilities of the phagemid upon large-scale growth. The resulting plasmids contained tandem promoters (*tac* from pKK223-3 and *trc* from the *Eco*RI–*Pst*I phagemid fragment), which allowed the production of 50–80 mg of protein per liter of cells. The expression vectors were used to transform *Escherichia coli* strain DF502, which is a $strep^R$,tpi^- strain that was kindly provided by D. Fraenkel and has been previously described (Straus & Gilbert, 1985).

Pure protein was prepared by growing the bacterial transformants in a final volume of 10 L of M63 salts (Miller, 1972) containing casamino acids (0.5% w/v), glucuronolactone (0.4% w/v), glycerol (0.1% w/v), $MgSO_4$ (1 mM), thiamine

(1 mg/mL), L-histidine (80 mg/L), streptomycin (100 mg/L), and ampicillin (200 mg/L). Cells were harvested after 12–20 h by centrifugation at 3000*g*. The cells were lysed in a continuous flow French pressure cell (Aminco, Urbana, IL), and the lysate was centrifuged at 8500*g* for 1 h to remove cell debris. The ammonium sulfate fraction from 55% to 90% saturation was collected and dialyzed overnight against TE buffer [10 mM Tris-HCl (pH 7.8) containing EDTA (1 mM)]. The crude protein was loaded onto a column (300 mL) of QAE Sephadex A-120 equilibrated with TE buffer and eluted with a linear gradient (1 L to 1 L) of KCl (0–300 mM). The proteins were finally purified on a MonoQ 10/10 column using the same gradient. The purity of the proteins was assessed by silver staining of overloaded 15% sodium dodecyl sulfate–polyacrylamide gels (Laemmli, 1970). Concentration of the proteins was afforded by Centriprep and Centricon concentration (Amicon, Danvers, MA).

Two other mutant isomerases besides K12M were prepared using similar methods. These were K12R, by using the oligonucleotide 5′-CC GTT TAA TCG AAA GTT ACC-3′, and K12H, by using the oligonucleotide 5′-CC GTT TAA GTG AAA GTT ACC-3′. For these two proteins, 200 mL of cell culture was prepared, and the ammonium sulfate precipitation step in the purification scheme was substituted by passage of the crude protein through a plug of DE-52 (Whatman, Kent, England). The resin was prepared by equilibration in 10 mM Tris-HCl buffer (pH 8.0) containing EDTA (1 mM), β-mercaptoethanol (1 mM) (buffer A) containing KCl (500 mM), and then equilibration in buffer A. A plug of DE-52 (approximately 6 mL) was prepared in a 30-mL syringe, and the protein was loaded onto the resin in buffer A. The isomerase protein was eluted from the plug of DE-52 with 15 mL of buffer A containing 150 mM KCl. The partially purified isomerase was desalted by ultrafiltration into buffer A and purified by MonoQ as described for the K12M mutant isomerase.

Isoelectric Focusing. The mutant K12M isomerase was analyzed by isoelectric focusing on an LKB Multiphor apparatus (Pharmacia LKB) at 4 °C. The gel was an Ampholine PAGplate (pH range 4–6.5) (Pharmacia LKB, Piscataway, NJ) and was focused for 3 h at 4.8 W to a maximum voltage of 1400 V. Isomerase activity of the proteins within the isoelectric focusing gel was assayed according to the method of Nickbarg (1988). The staining solution contained 100 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM β-mercaptoethanol, 0.4 mM DHAP, 35 mM Na_3AsO_4, 7 μg/mL glyceraldehyde-3-phosphate dehydrogenase, 50 μg/mL nicotine adenine dinucleotide, 35 μg/mL phenazine methosulfate, and 200 μg/mL nitro-blue tetrazolium. The gel was first stained for isomerase activity by placing a filter paper saturated in staining solution on top of the gel for 5–10 min. The gel was then fixed and stained for protein by Coomassie blue.

FTIR Spectroscopy. Infrared absorbance spectra were recorded using an FTS-40 instrument (Digilab, Cambridge, MA) equipped with a temperature-controlled micro circle cell (Spectra-Tech, Stamford, CT). The sample compartment was purged with dry N_2 for several hours after installation of the circle cell and before injection of the protein samples to minimize water vapor absorbance in the spectra. The spectral resolution was 2 cm^{-1}, and the number of scans accumulated per Fourier transform was 1024. The time required for data acquisition was 20 min. The samples were cooled to 8 °C. The protein samples were first concentrated to 200–300 mg/mL and then exchanged into deuterated buffer that had been prepared by lyophilizing 100 mM Tris-HCl buffer (pH 7.6) containing EDTA (1 mM) and then dissolving this residue in D_2O. The buffer had a pD of approximately 8. The volume of sample required to fill the circle cell and connecting tubing was approximately 60 μL. The circle cell was washed exhaustively with buffer between each filling with sample, cleaned daily with a dilute solution of low-foaming detergent, and finally washed extensively with H_2O. Protein (70 μL, 10 mN final concentration of active sites) was mixed with substrate (4 μL, 14 mM final concentration) immediately before the sample was injected into the circle cell. In one case, the K12M mutant protein was incubated with substrate overnight prior to injection into the circle cell to detect any slowly occurring processes. No differences were observed between this sample and those prepared immediately before analysis.

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

Determination of Kinetic Constants. Steady-state kinetic constants for K12M, K12M·G15A, and K12R were determined at 30 °C under the following conditions. A stock solution (200 μL) of 1 M triethanolamine hydrochloride buffer (pH 7.6) containing EDTA (100 mM) was added to a cuvette, along with DL-glyceraldehyde 3-phosphate (GAP) (25–300 μL of a 75 or 77 mM solution, prepared as described above and then brought to pH 7.4 with 5 N NaOH), and diluted with H_2O (to 1 mL). NADH (140 μg) was added from a stock solution in H_2O, followed by glycerol-3-phosphate dehydrogenase (10 μL, free of triosephosphate isomerase as described above) and a sample of mutant isomerase (25 μL of a solution containing from 0.05 to 1 mg/mL, depending on the activity of the mutant). The inhibition constant for the intermediate analog, phosphoglycolohydroxamate, was determined under the same conditions, but with an inhibitor concentration, of 0, 0.96, or 1.9 mM.

Steady-state kinetic constants for K12H triosephosphate isomerase at pH 6.1 were determined at 30 °C under the following conditions. A stock solution (200 μL) of 1 M MES buffer (pH 6.1) containing EDTA (100 mM) was added to a cuvette, along with DL-glyceraldehyde 3-phosphate (GAP) (25–300 μL of a 91 mM solution, prepared as described above and then brought to pH 6.1), and diluted with H_2O (to 1 mL). The addition of NADH, glycerol-3-phosphate dehydrogenase, and mutant isomerase was as described above.

X-ray Crystallography. The double mutant TIM, K12M·G15A, was dialyzed against 200 mM Tris-HCl buffer (pH 6.8) containing EDTA (1 mM) and β-mercaptoethanol (1 mM) with four changes of buffer in Centricon concentrators. Protein crystals were grown at room temperature in 100-μL portions containing TIM (20 mg/mL) and phosphoglycolohydroxamate (1.5 mM) in one-half dram vials loosely fitted with cork stoppers. Crystals appeared after 5–10 days. Attempts to crystallize the single mutant TIM, K12M, were unsuccessful.

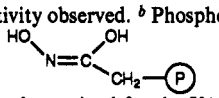
RESULTS

Steady-State Kinetic Constants. The steady-state kinetic constants for the yeast isomerase mutants K12M, K12M·G15A, K12R, and K12H have been determined and compared to that of the wild-type enzyme. These data are listed in Table 1. Both the K12M and K12M·G15A mutants have little or no activity. It is interesting to note that the K_m

Table 1: Steady-State Kinetic Constants for K12M, K12M·G15A, K12R, and K12H Mutant Triosephosphate Isomerases

enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	K_i for PGH[b] (mM)
wild type	8700	1.5	5800	0.015
K12M[a]	0.018	0.6	0.03	ND[c]
K12M·G15A[a]	0.125	1.5	0.08	ND[c]
K12R	48	33	1.45	4.3
K12H (pH 6.1)	6.3	23	0.3	0.12
K12H (pH 7.5)	0.89	61	0.015	>10

[a] The kinetic constants for the TIM mutants K12M and K12M·G15A are due to contamination with a small amount of wild-type TIM, the level of which varied depending on the preparation of the enzyme. The K_m's reported for these two mutants are equivalent within experimental error; the determination of kinetic constants for these mutants was inaccurate due to the low level of activity observed. [b] Phosphoglycolohydroxamate:



[c] The K_i for PGH was not determined for the K12M and K12M·G15A mutant isomerases because the activity we observed in these preparations was due to contaminating wild-type isomerase.

determined for the K12M mutant enzyme is close to that for the wild-type, and the observed activity can be accounted for by the presence of a contaminating level of wild-type isomerase of 0.03%. This corresponds to a translational error rate of 1 in 3×10^4 bases, which is close to the measured rate of translational error in *E. coli* (Schimmel, 1989). It proved possible to demonstrate that the low observed activity is indeed due to the presence of a small amount of wild-type enzyme by isoelectric focusing gel electrophoresis followed by staining for isomerase activity (see below).

The data in Table 1 also indicate that mutants with a positively charged side chain at position 12 all have some activity. The K12R mutant is about 4000-fold less active than the wild-type isomerase, but is still quite a proficient catalyst. Since arginine is a larger side chain than lysine, it seems likely that the increase in K_m is due to steric crowding of the active site. The extremely high K_i for PGH that has been measured for this mutant is consistent with this hypothesis.

Substitution of lysine-12 with histidine is much more damaging to the catalytic efficiency of the isomerase than is substitution with arginine. This result is not unexpected given that, in the structure of the wild-type enzyme, this lysine is fully extended. Substitution with histidine places the positively charged group (now imidazolium) farther away from the substrate than the ammonium group of a fully extended lysine. Interestingly, the K12H mutant has almost no activity at pH 7.5, but can be "turned on" by lowering the pH to 6.1. At pH 7.5, the catalytic activity is reduced 400000-fold from the wild type, but its activity is increased by about 20-fold at the lower pH. Further, at pH 6.1 PGH is a respectable inhibitor, indicating that the substrate binding site is relatively unperturbed by the substitution of histidine for lysine. It should be noted that the pH maximum for the wild-type isomerase is 7.4, so that the increase in activity of this mutant isomerase at low pH is quite different from the behavior of the wild-type enzyme (Plaut & Knowles, 1972). These data also support the hypothesis that a positively charged group is required for proper substrate binding.

Isoelectric Focusing. The mutant isomerases that contain the K12M mutation were analyzed by isoelectric focusing in the pH range of 4.0–6.5. Wild-type TIM had a p*I* of 5.5, and the K12M and K12M·G15A mutant proteins each had a p*I* of 5.25. When relatively large amounts of either mutant isomerase (15–30 μg) were loaded on the gel, protein was visible at a p*I* of 5.25 but no activity was observed at this p*I*.

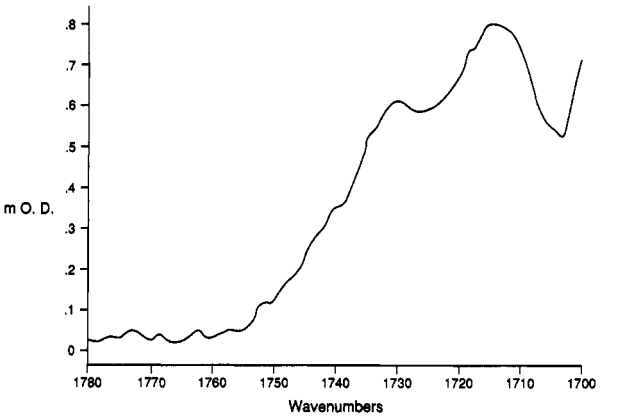


FIGURE 2: FTIR spectrum of wild-type yeast TIM (10 mN) with DHAP (7 mM). Spectral subtraction has been used to remove the absorption of unbound substrate from the spectrum. Two bands are observed at 1713 and 1732 cm^{-1}.

No protein was observed at a p*I* of 5.5 by Coomassie blue staining, but TIM activity was visible at this p*I*. For these experiments, we calculated how much wild-type isomerase activity we expected to find in the mutant isomerase preparations on the basis of the measured kinetic constants, and as a control, we loaded wild-type protein on the gel in the amounts we expected to see in the mutant isomerase preparation. The amount of activity we observed at a p*I* of 5.5 in the lane containing the mutant isomerase preparation appeared equivalent to the amount of activity we observed in the lane containing the control wild-type protein. These results clearly indicated that the activity observed in the K12M mutant protein was due to contaminating wild-type enzyme and not to an intrinsic weak activity of the K12M mutant. Since we were very careful to avoid contamination of the wild-type protein in the preparation of the mutant isomerases, and since our calculations of activity are consistent with translational error rates, we presume that the contaminating wild-type isomerase was produced by translational error and is not the result of contamination in some step of the protein preparation.

Binding of Substrates to the K12M Mutant by FTIR. The FTIR spectrum of wild-type yeast TIM with dihydroxyacetone phosphate bound is shown in Figure 2. This spectrum is the result of subtraction of a spectrum of both unbound substrate as well as that of free enzyme. These subtractions do not alter the value of ν_{max}, since the absorbance bands for the bound substrate are much narrower than those of free substrate or free enzyme. Figure 3 shows the FTIR spectrum of the K12M mutant in the presence of DHAP. The absorption of free enzyme has been subtracted from each spectrum. A different amount of DHAP has been subtracted from each spectrum to best visualize any narrow bands due to bound substrate. These spectra show that DHAP does not bind in the active site of the K12M mutant isomerase. Preincubation of either GAP or DHAP with the K12M mutant overnight before FTIR analysis gave identical results. On the basis that the monoanionic substrate analog, dihydroxyacetone sulfate (DHAS), might bind to the K12M mutant (each carrying one less charge than the parent species), the infrared spectra of mutant and wild-type enzymes with this ligand were measured. Dihydroxyacetone sulfate has an infrared absorbance spectrum very similar to that of DHAP, except that its absorbance maximum is at 1742 cm^{-1} while that of DHAP is 1732 cm^{-1}. The spectra looked identical to those shown in Figure 2, except the absorbance maximum was shifted to 1742 cm^{-1}, and we concluded that no binding of the sulfate to the enzyme was observed. Thus, neither doubly nor singly anionic

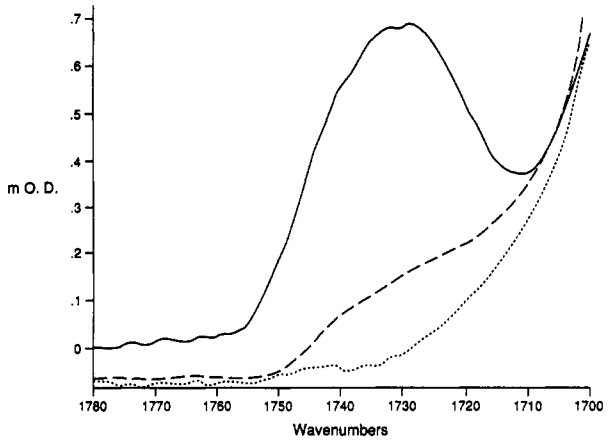


FIGURE 3: FTIR spectra of the K12M mutant TIM (10 mN) with DHAP (7 mM). The solid line is the spectrum before subtraction of the absorption of unbound substrate. This spectrum is close to that for DHAP: it has a broad absorbance centered at 1732 cm^{-1}. The dashed line is the spectrum from which most of the unbound substrate has been subtracted, and the dotted line is the spectrum from which all of the unbound substrate has been subtracted. Data from the experiment using DHAS as the substrate resulted in spectra that were identical to these, except that the DHAS band is observed at 1742 instead of 1732 cm^{-1}.

substrates will bind to the active site of mutant isomerases in which the lysine at position 12 has been substituted for methionine.

Crystallization of the K12M·G15A Mutant with PGH. To determine whether any large structural perturbations were generated by the K12M mutation, we crystallized the mutant isomerase in the presence of the intermediate analog, PGH. Large crystals of the double mutant protein, K12M·G15A, were rapidly and reproducibly obtained. These crystals diffracted to a resolution higher than 1.9 Å, and the structure was determined (the details of the structure are presented in an accompanying article). The surprising features of this structure are that the protein is in the "loop open" conformation and no PGH is bound at the active site. Otherwise, the structure is essentially identical to that of the wild-type protein structure that was determined from crystals grown in the absence of PGH (Lolis et al., 1990). This result confirms that lysine-12 does not play a major role in the maintenance of the structure of the protein, but is important for substrate binding.

DISCUSSION

Mutation of the conserved residue, lysine-12, in the active site of triosephosphate isomerase destroys the catalytic activity of the enzyme. Substitution of this residue with another that is positively charged (e.g., arginine) or one that can be positively charged (e.g., histidine at low pH) results in a protein with measurable activity, but alteration of this residue to an uncharged amino acid (e.g., methionine) results in a protein with no measurable activity. These results suggest that a positive charge at the active site of TIM is essential for enzymatic activity.

Preparations of the K12M mutant isomerase do have detectable activity, but this activity can be traced to small amounts of contaminating wild-type protein. The wild-type protein was easily separated from the mutant protein by isoelectric focusing in native gels that were stained for isomerase activity. The activity was found to focus at the isoelectric point of the wild-type enzyme. Strict precautions were taken to ensure that the glassware used in the purification was acid-washed and that the protein was expressed in a strain of *E. coli* that cannot produce the enzyme; thus, we conclude that the small amount of wild-type protein is due to translational error. This experience reemphasizes the caveat of Schimmel (1989) that translational error may contribute to the activity observed in weakly active mutant protein preparations, such as those observed in "alanine-scanning" experiments.

To determine the role of lysine-12 in the action of the isomerase, we have examined the basis of the defect in the K12M mutant enzyme. The lysine cannot be responsible for polarizing the carbonyl groups of the substrate, since that role is known to be performed by histidine-95 (Komives et al., 1991). Thus, in the H95Q mutant enzyme, no substrate carbonyl polarization is evident by FTIR, despite the fact that lysine-12 is present and that the substrate analog, PGH, is bound in exactly the same place in the mutant crystal structure as in the wild type. The experiments reported in this article directly implicate lysine-12 in substrate binding. First, in the K12M mutant isomerases, no bound substrate can be observed by FTIR even at high concentrations of substrate and overnight incubations. Second, the K12M mutant isomerase does not bind DHAS, a monoanionic substrate analog. This result suggests that the lysine ammonium group changes the overall electrostatic character of the active site and does not merely neutralize one of the negative charges on DHAP. Third, when crystals of the K12M·G15A mutant isomerase are grown in the presence of PGH under conditions where crystals of *liganded* complex are obtained for the wild-type enzyme, we found that the active site in the mutant crystal structure was open, and no substrate analog was observed in the active site (Joseph-McCarthy et al., 1994).

Finally, we have tested the hypothesis that *any* positive charge would be able to replace the lysine residue in the active site. For these experiments, we substituted lysine-12 with arginine or histidine. Substitution with arginine is not sterically ideal: arginine is larger than lysine and is expected to extend too far into the substrate binding pocket. Substitution with histidine is not ideal either, both because the positive imidazolium side chain would not extend as far into the substrate binding pocket as the ammonium group of lysine and because the pK_a of histidine is nearer to neutrality. Nonetheless, both the K12R and the K12H mutant isomerases showed measurable, low activity. Notably, the K12H mutant showed a 20-fold increase in catalytic activity at pH 6.1 (where a higher proportion of positively charged histidine is expected) as compared to pH 7.5. The K12R mutant enzyme displays poorer substrate binding and also binds the reaction intermediate analog (PGH) less tightly. Interestingly, every amino acid substitution that provided a positive charge restored enzyme activity. These results provide strong evidence that the role of the lysine residue is simply to provide a positive charge at the active site. Taken together, these experiments demonstrate the importance of the overall electrostatic character of an active site and delineate the role of the conserved lysine-12 at the active site of triosephosphate isomerase.

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