Crystallographic Analysis of the Complex between Triosephosphate Isomerase and 2-Phosphoglycolate at 2.5-Å Resolution: Implications for Catalysis†,‡

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Received June 30, 1989; Revised Manuscript Received December 11, 1989

ABSTRACT: The binding of the transition-state analogue 2-phosphoglycolate to triosephosphate isomerase from yeast has been investigated crystallographically. An atomic model of the enzyme–inhibitor complex has been refined against data to 2.5-Å resolution to a final R factor of 0.18. The interactions between the inhibitor and enzyme have been analyzed. The inhibitor forms hydrogen bonds to the side chains of His 95 and Glu 165. The latter hydrogen bond confirms that Glu 165 is protonated upon PGA binding. The structure of the complexed enzyme has been compared to that of the unbound form of the enzyme, and conformational changes have been observed: the side chain of Glu 165 moves over 2 Å and a 10-residue flexible loop moves over 7 Å to close over the active site. Spectroscopic results of phosphoglycolic acid binding to triosephosphate isomerase that have been amassed over the years are also explained in structural terms. The implications for catalysis are noted.

Triosephosphate isomerase (EC 5.3.1.1) is a glycolytic enzyme that catalyzes the interconversion of D-glyceraldehyde 3-phosphate (GAP)[1] and dihydroxyacetone phosphate (Figure 1). The enzyme from yeast is a dimer of identical subunits, each of molecular weight about 26 000. The crystal structure (Banner et al., 1975; Alber et al., 1981a; Wierenga et al., 1987), free energy profile (Albery & Knowles, 1976; Nickbarg & Knowles, 1988), and characterization of site-specific mutants (Raines et al., 1986; Ahern et al., 1987; Casal et al., 1987; Nickbarg et al., 1988) from various organisms have been reported.

It is the hope in this laboratory to understand the structural basis of catalysis using crystallography, site-directed mutagenesis, kinetic experiments, and computer simulation of the reaction. As part of this program, we have undertaken to determine high-resolution structures of the uncomplexed enzyme [preceding paper in this issue (Lolis et al., 1990)] and the enzyme complexed with catalytically relevant ligands. In this report, we describe the structure of the complex between triosephosphate isomerase and 2-phosphoglycolate and make comparisons with the unbound form of the enzyme. The crystallographic results are also used to interpret various NMR, UV, and pH dependence experiments (Campbell et al., 1978, 1979; Jones & Waley, 1979).

2-Phosphoglycolate has been proposed to be a transition-state analogue of TIM even though it is one methylene group shorter than any chemical species found on the reaction coordinate (Figure 1). The absence of a number of atoms relative to the true transition state does not prevent PGA from binding to the enzyme over 100 times more tightly than either DHAP or GAP, which have K_m's on the order of 1 mM. Although tight binding of an inhibitor provides powerful support for the designation of transition-state analogue, it should not be the only evidence. At least one tight-binding inhibitor (methotrexate) that was once thought to be a transition-state analogue of an enzyme (dihydrofolate reductase) has subsequently been found to be opportunistic, taking advantage of binding interactions that have little relevance to catalysis (Mathews et al., 1978; Williams et al., 1979). Although PGA replaces a methanol group with a much smaller oxygen atom and is therefore not a conventional transition-state analogue where only one atom might be changed, it is also not believed to be opportunistic. As shown in Figure 1, PGA is thought to have the stereochemistry and charge configuration that mimics part of the transition state of the catalytic step that converts DHAP to the enediolate intermediate. For these reasons, the interactions between PGA and TIM have been studied extensively by using pH experiments (Hartman et al., 1975), UV spectrophotometry (Jones & Waley, 1979), and NMR (Campbell et al., 1978, 1979). Phosphoglycolohydraxamate (PGH), an inhibitor that emulates the enediolate intermediate, has also been synthesized and studied (Collins, 1974). Its structure complexed to TIM has recently been investigated to 1.9-Å resolution (R. C. Davenport, B. Seaton, G. A. Petsko, and D. Ringe, unpublished results).

Materials and Methods

The cocrystallization of yeast TIM and PGA has been previously reported (Alber et al., 1981b). Yeast triosephosphate isomerase (Sigma Chemical Co., type I) was exhaustively dialyzed against a solution of 0.2 M Tris (pH 6.8) containing 1 mM EDTA and 1 mM mercaptoethanol. Batch methods were used to crystallize the enzyme. Poly(ethylene glycol) 4000 (J. T. Baker Chemical Co.) was added to a final concentration of 15–20% to a solution containing 20 mg/mL yeast TIM and 1.5 mM phosphoglycolic acid (Sigma). At this point the solution appeared slightly cloudy; a small amount (5–10 μL) of either water or buffer was added to redissolve the enzyme. Usable crystals (0.6 mm × 0.5 mm × 0.4 mm) were obtained in about a month at room temperature. The crystals have the symmetry of the space group $P2_1$ with one dimeric molecule in the asymmetric unit. The unit cell of the crystal has dimensions a = 74.35 Å, b = 83.97 Å, c = 38.67 Å, and β = 99.70°. It is isomorphous with the monoclinic crystal form obtained on cocrystallization of yeast TIM with

† This work was supported by grants from the National Institutes of Health. We also acknowledge financial support from Berlex Laboratories.
‡ Crystallographic coordinates have been submitted to the Brookhaven Protein Data Bank.

[1] Abbreviations: DHAP, dihydroxyacetone phosphate; GAP, D-glyceraldehyde 3-phosphate; NMR, nuclear magnetic resonance; PGA, 2-phosphoglycolate; PGH, phosphoglycolohydroxamate; rms, root mean square; TIM, triosephosphate isomerase; UV, ultraviolet.

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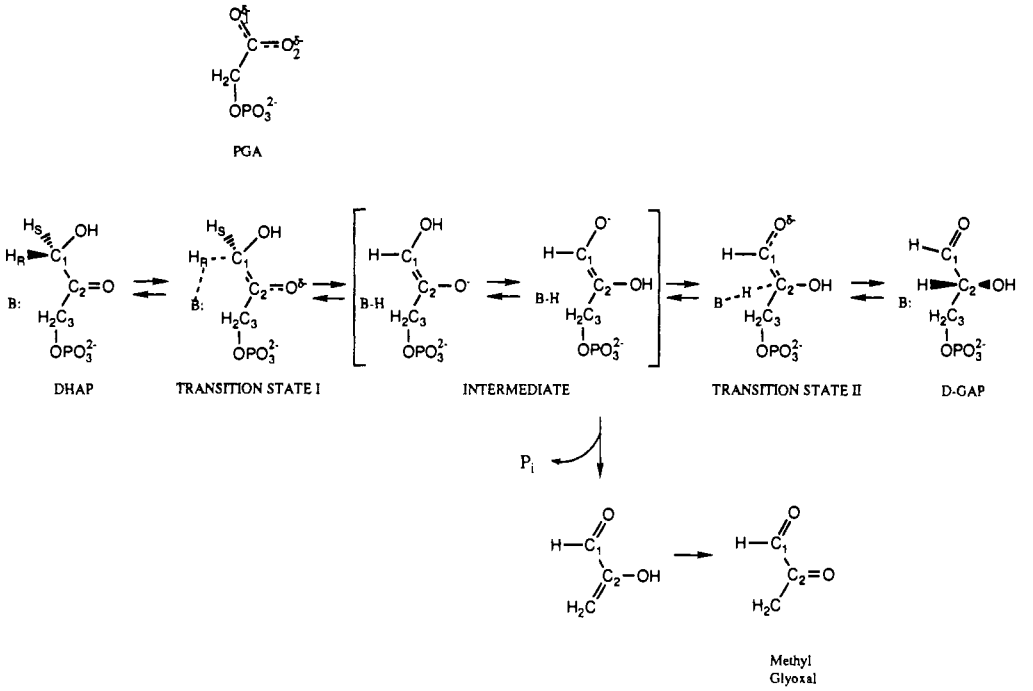

FIGURE 1: Reaction catalyzed by triosephosphate isomerase, including the transition states for proton transfer. The subscripts on the carbon atoms refer to the numbering scheme for the molecule. For substrates, transition states, and intermediates, the numbering of the oxygens corresponds to the numbering of the carbon atoms to which they are bonded. The numbering of the PGA carboxylate oxygens is also shown. "B:" refers to the catalytic base. Although the intermediate is depicted as a kinetically indistinguishable pair of enediolates, it is possible that it is really a *cis*-enediol. The phosphate elimination side reaction is shown proceeding from one of the enediolates. PGA is shown directly above the transition state for which it is thought to be an analogue.

PGH (Davenport et al., unpublished results).

The crystals were mounted at room temperature in a quartz capillary tube with a column of mother liquor on each side to keep the crystal from drying out. The tube was also sealed with mineral oil and wax. Data were collected on two crystals on a Nicolet P3 diffractometer at room temperature, with nickel-filtered copper Kα radiation. About 10000 reflections were collected from 44- to 3.0-Å resolution on one crystal, and 8300 reflections from 3.15- to 2.5-Å resolution on the other. The data were collected by the full integration method, scanning on ω for a total of 1°, counting for 30 s on the peak and 15 s on the background, which was collected 1° away from the calculated peak position. The intensities of five standard reflections, which were distributed through reciprocal space and were monitored during data collection, had decayed by 40% after about 110 h of data collection. The data were processed by using the PROTSYS package of programs (Petsko, unpublished results) on a VAX 11/750. Radiation damage could be corrected by an approximation of linear decay of intensity with exposure time using the standard reflections. An emperical method was used to correct for absorption (North et al., 1968). The reduced data from the two crystals were scaled and merged with an R_{merge} ($R_{merge} = \sum_{hkl}|I - \bar{I}|/\sum_{hkl}I$) of 8.4% for about 800 overlapping reflections. Sixty-four percent of the theoretical number of reflections had intensities twice the standard deviation and were kept for further work.

The coordinates of the protein atoms from the TIM–PGH complex (Davenport et al., unpublished results), which is isomorphous to the TIM–PGA crystal form, were used as the initial model. The structure (TIM–PGH) was solved by a combination of isomorphous and molecular replacement. Ethyl mercuric phosphate was used to make a heavy metal derivative. In the native structure (Lolis et al., 1990), which is not isomorphous to the TIM–PGH (or –PGA) crystal form, the mercury atoms are known to bind to Cys 126 in both subunits of the dimer. An incompletely refined model of the native enzyme structure was then rotated into the TIM–PGH unit cell such that Cys 126 from each subunit was placed into the two Patterson-defined mercury positions of the TIM–PHG asymmetric unit. Finally, rotation of the dimer about the axis defined by the vector between the two mercury atoms, combined with an R factor analysis, revealed the correct orientation of the TIM dimer in this unit cell.

A $2F_o - F_{c,omitPGH}$ Fourier map was calculated by deleting the bound PGH of the yeast TIM–PGH structure (Davenport et al., unpublished results) for the structure factor and phase ($e^{2\pi i\alpha omitPGH}$) calculation, and using the observed structure factors for the yeast TIM–PGA crystal. After minor adjustments, the structure without the coordinates for PGA was refined with the restrained least-squares program of Hendrickson and Konnert (Hendrickson, 1985). The initial cycle of refinement included all data; the R factor was 33.1%. Six subsequent cycles in the first round of refinement with data from 10.0- to 2.8-Å resolution yielded an R factor of 24.5%. After a number of sessions involving rebuilding and refinement, the R factor dropped to 18.5% for reflections from 5.0 to 2.5 Å. Addition of coordinates for PGA and 24 waters (which were placed in difference density of over 2 standard deviations) and additional refinement yielded a final structure whose R factor was 16.7%. At this stage, the reflections from 10.0- to 5.0-Å resolution were added in the refinement. The scattering contributions from bulk solvent were modeled by a method using the Babinet principle (Fraser et al., 1978). The final R factor for 10355 reflections between 10.0- and 2.5-Å resolution is 18%. Refinement statistics are given in Table I.

RESULTS

It has been previously observed that the native unbound crystals of triosephosphate isomerase that were soaked in solutions containing either substrates or inhibitors showed sub-

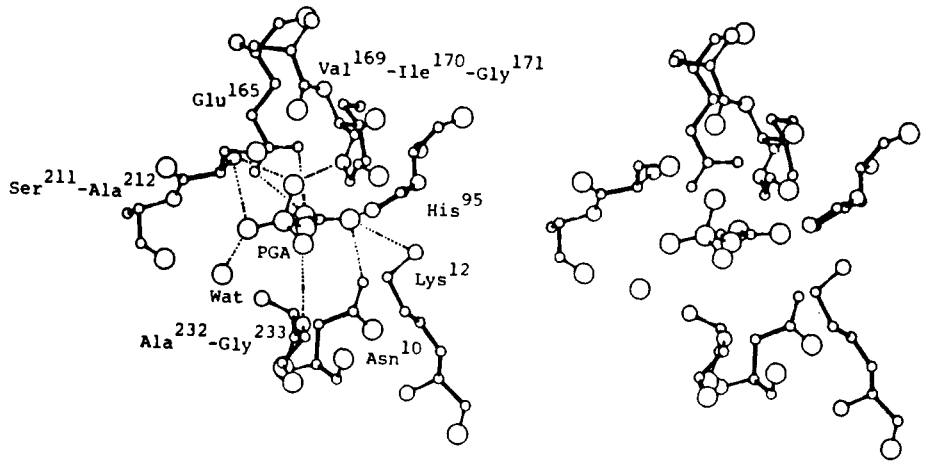

FIGURE 2: Stereoscopic view of PGA in the active site of subunit 1 surrounded by residues that are within 3.5 Å. All hydrogen bonds to PGA are shown as dotted lines.

Table I

Refinement Statistics		restraints applied, σ (Å)	deviations obsd, Δ (Å)[a]
no. of protein atoms	3766		
no. of inhibitor (PGA) atoms	18		
no. of water molecules	24		
bond distance		0.030	0.026
angle distance		0.040	0.052
planar 1–4 distance		0.052	0.058

Resolution Breakdown				
resolution (Å)	no. of reflections	% of data	shell R factor	sphere R factor
10.0–4.80	1754	81.2	0.236	0.236
4.80–3.90	1615	79.1	0.152	0.192
3.90–3.40	1625	72.6	0.159	0.182
3.40–3.10	1385	65.7	0.176	0.181
3.10–2.80	1939	62.7	0.173	0.180
2.80–2.50	2037	42.7	0.164	0.177
10.0–2.50	10355	63.1		0.177

[a] These Δ values are rms deviations from the corresponding values for ideal groups derived from small molecular structural studies (Sielecki et al., 1979).

stantial changes in the diffraction pattern. In most cases, the crystals cracked, resulting in both weaker diffraction and unit cell changes (Johnson & Wolfenden, 1970; Alber, 1981). Conditions were found that allowed the diffusion of phosphate or DHAP into chicken TIM crystals to be studied, but only to 6-Å resolution (Rivers, 1977; Phillips et al., 1977). In order to overcome these problems and observe the bound enzyme complex at higher resolution, TIM was cocrystallized with PGA. These crystals were of a different crystal form from those of the uncomplexed enzyme but were isomorphous with cocrystals of yeast TIM and PGH. The details describing the solution of the yeast TIM–PGH crystal form are given elsewhere (Davenport et al., unpublished results).

Comparison of the Two Subunits in the Dimer. The accuracy of the atomic coordinates in this structure can be assessed by comparison of the two subunits that comprise the asymmetric unit of the crystal. A least-squares superposition of the Cα's of the two subunits leads to an rms difference of 1.0 Å. This is a much higher number than would normally be expected for two identical subunits. [Structures with sequences that differ by 50% have a similar rms difference. A least-squares comparison of 490 Cα's between the uncomplexed chicken and yeast triosephophate isomerase, which have 53% sequence identity, also yields an rms difference of 1.0 Å (Lolis et al., 1990)]. Close inspection of the superimposed TIM–PGH subunits reveals major differences at residues 2, 55, and 56. As with most protein structures, the terminal ends are highly flexible and are hard to place in the electron density map, so it is not unexpected that there is a large difference at Ala 2. Residues 55 and 56 (and the corresponding residues in the other subunit) are in a loop at the surface of the protein. These two loops on the superimposed subunits are over 6 Å apart. Such a large difference might indicate that one (or both) of the loops is misplaced. This interpretation is supported by dihedral angles for the loop in the second subunit that are outside the Ramachandran limits. However, $F_o - F_c$ delete maps indicate that the loop in each subunit is correctly defined by density. The only obvious difference that might explain the two distinct conformations is a rather long (4-Å) electrostatic interaction in the second subunit between the side chain of Lys 56 and a carboxylate oxygen of a symmetry-related molecule; this interaction is absent in the first subunit. We therefore concluded that the different conformation of this loop in the two subunits is due to crystal contacts.

In order to eliminate the effect due to the crystal contact or to the flexibility of the amino-terminal end of the protein, residues 2, 55, and 56 from both subunits were left out of the least-squares superposition calculation. The rms difference of the Cα's between the two subunits dropped to 0.6 Å. The rms difference between all atoms in the two active sites (including residues 10, 12, 95–97, 126, 165, 167–176, and PGA) is also 0.6 Å. Although these comparisons must certainly include other residues that are flexible, they cannot be identified from the experimental data. Individual B factors, which have been correlated with flexibility (Petsko & Ringe, 1984), were not refined in this structure due to low overdeterminancy of the data (less than 11 000 reflections for almost 4000 atoms). We can only conclude from this analysis that 0.6 Å seems to be a conservative estimate for the average error in the atomic coordinates for this structure.

The PGA-Bound Active Site. The enzyme makes a substantial number of hydrogen bonds to PGA in the active site (Figure 2). The O_1 carboxylate oxygen of PGA (for the numbering scheme of the atoms, see Figure 1) forms hydrogen bonds to the protonated (see below) Oϵ2 (2.9 Å)[2] carboxylate

[2] Only the hydrogen bonds that are common to both active sites are noted in this paragraph. An interaction is defined as a hydrogen bond if the distance between the two polar atoms is less than 3.3 Å. The distance that appears in parentheses is the average of both subunits. For a complete list of all hydrogen bonds in the two active sites, Table II should be consulted.

Table II: Hydrogen Bond Distances between Non-Hydrogen Atoms of PGA and TIM[a]

hydrogen bond	distance (Å)
O_1 249–Nδ2 Asn 10[b]	3.2
O_1 249–NZ Lys 12	3.0
O_1 249–Nε2 His 95	2.7
O_2 249–Nε2 His 95[b]	3.1
O_2 249–Oε1 Glu 165	3.2
O_2 249–Oε2 Glu 165	2.7
O_4 249–N Gly 171	2.6
O_4 249–N Ser 211	2.7
O_4 249–Oγ Ser 211[c]	3.3
O_5 249–N Gly 171[c]	3.2
O_5 249–N Gly 233[b]	3.3
O_6 249–N Ser 211[b]	3.1
O_6 249–N Gly 232[c]	3.1
O_6 249–O Wat 640[b]	2.8
O_6 249–O Wat 641	2.4
O_6 249–O Wat 643	2.5

[a] The distance is the average of both subunits unless otherwise noted. [b] This hydrogen bond is found only in subunit 1. [c] This hydrogen bond is found only in subunit 2.

oxygen of Glu 165. The O_2 carboxylate oxygen of PGA forms hydrogen bonds to the NZ (3.0 Å) atom of Lys 12 and the Nε2 (2.7 Å) of His 95. The terminal phosphate oxygens are hydrogen bonded to the backbone amide nitrogens of Gly 171 (2.6 Å) and Ser 211 (3.0 Å) and a water molecule. A complete list of active site hydrogen bonds is given in Table II.

The bridging phosphate oxygen does not make any hydrogen bonds at all. This is particularly surprising in view of the fact that the phosphonate analogues of DHAP and GAP, where the bridging oxygen is replaced by a methylene group, are poor substrates for the enzyme. Whereas the phosphonate analogue of D-GAP, D-2-hydroxy-4-phosphonobutyraldehyde [CHOC$H(OH)CH_2PO_3H_2$], has an enzyme-catalyzed rate constant that is 800-fold lower than natural substrate (Belasco et al., 1978), the DHAP analogue, (4-hydroxy-3-oxobutyl)-phosphonic acid [$CH_2(OH)C(O)CH_2CH_2PO_3H_2$], hardly binds to the enzyme at a concentration as high as 10 mM (Dixon & Sparkes, 1974). Similarly, the phosphonate analogue of PGA, 3-phosphonopropionate ($CO_2HCH_2CH_2PO_3H_2$), has an affinity about 10^3 lower for TIM than PGA (Wolfenden, 1970; Waley, 1973). All these data led to speculation that interactions with the bridging oxygen, presumably by Lys 12, accounted for the specificity of this enzyme. The ε-nitrogen of Lys 12, however, is involved in hydrogen bonds with one of the carboxylate oxygens of PGA and with Glu 97 over 4.0 Å away from the bridging oxygen. What determines the specificity for the natural substrates over their phosphonate analogue may have little to do with hydrogen bonding and more to do with the preference of methylene groups for the staggered conformation. As can be seen from Figure 2, the bridging oxygen adopts a conformation that is planar with the PGA carboxylate group. If upon binding to enzyme, the substrate is strained toward a similar conformation, the bridging oxygen and the O_2 oxygen would be distorted from a staggered to an eclipsed conformation. With a phosphonate analogue, the energetic cost of moving a methylene group from a staggered to an eclipsed conformation might be too high for binding and catalysis to occur (Dixon & Sparkes, 1974).

From NMR experiments PGA is known to bind to TIM as a trianion, with two negative charges on the phosphate and a negative charge on the carboxylate (Campbell et al., 1978). On the basis of pH titration experiments, it has been found that a proton is taken up by the enzyme upon PGA binding (Jones & Waley, 1979); this phenomenon is not observed with the binding of substrate or any other inhibitor (Campbell et al., 1979). In view of these results, it is appropriate to discuss the ionization state of the active site and the potential electrostatic interactions between enzyme and substrate. The presence of the negatively charged carboxylate of PGA in the active site can be assumed to be the cause of the proton uptake by the enzyme. To try to deduce which atom has become protonated, the structure of the active site has been analyzed. The only residues that interact with the carboxylate group of PGA are Lys 12, His 95, and Glu 165. Lys 12, in the unbound enzyme, is believed to be positively charged as it interacts with the carboxylate of Glu 97. Therefore, Lys 12 is incapable of accepting a proton, nor does it seem plausible that it relays a proton to Glu 97. It is also unlikely that the ionization state of the imidazole of His 95 changes upon binding of PGA. In the unbound enzyme, the imidazole Nδ1 nitrogen acts as a hydrogen bond acceptor to the backbone amide nitrogen of Glu 97, while the Nε2 nitrogen is hydrogen bonded to a water molecule in the active site (preceding paper in this issue). It is most likely in this arrangement that only the Nε2 nitrogen of the imidazole is protonated. In the bound form, the PGA displaces the water molecule and is now the hydrogen bond acceptor to the imidazole Nε2 nitrogen. The Nδ1 nitrogen continues to be hydrogen bonded to the backbone amide nitrogen. Glu 165, on the other hand, changes from being hydrogen bonded to the hydroxyl oxygen of Ser 96 to being hydrogen bonded to the carboxylate oxygen of PGA. Various experiments (Waley, 1972; Hartman et al., 1975; Belasco et al., 1978) have implicated a pK_a value below 5 for the Glu 165 carboxylate. It seems reasonable that Glu 165 becomes protonated to replace a potentially destabilizing electrostatic repulsion between its side-chain carboxylate and the anionic carboxylate of PGA with what is known to be a very strong bond between two carboxylates that interact through a proton (Jeffrey & Maluszynska, 1982). The replacement of the C_1 carbon atom of substrate by the O_1 oxygen of PGA presumably accounts for some of the additional binding energy exhibited by PGA relative to the substrates. This replacement changes the (Glu 165)–C–O^-···H–C–(substrate) interaction to the much stronger (Glu 165)–C–O–H···$^-$O–C–(PGA) interaction.

Conformational Change upon Binding of PGA. It has been known for twenty years that TIM undergoes a conformational change upon substrate or PGA binding (Johnson & Wolfenden, 1970). A 6-Å difference electron density map of chicken TIM crystals soaked with substrate first indicated the presence of large-scale motions around the active site (Rivers, 1977; Phillips et al., 1977). Subsequently, changes in the UV absorbance, which were attributed to tyrosine and tryptophan residues, were observed upon binding of ligands (Jones & Waley, 1979). Besides noting the specific interactions between TIM and PGA, we are now able to describe the nature of the conformational change in greater detail.

A least-squares superposition using only the Cα's of the unbound and bound form of yeast TIM reveals one significant change. A 10 amino acid loop, comprised of residues 167–176, has moved from an open conformation in the unbound form to a closed one with PGA in the active site. Some atoms in this loop move over 7 Å to form a single hydrogen bond and numerous van der Waals contacts with the inhibitor.

The only polar interaction between the loop and the PGA involves a hydrogen bond between the backbone amide nitrogen of Gly 171 and a phosphate oxygen. A superposition of only the atoms in the loop reveals that the side chains do not undergo any conformational changes during the motion

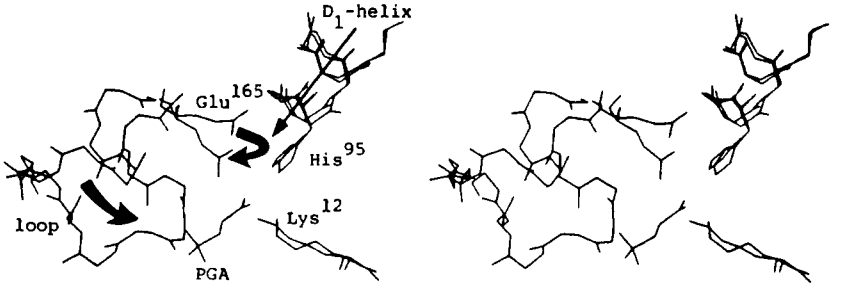

FIGURE 3: Conformational changes in the TIM active site upon binding of PGA. This view was generated from the orientation matrix between Cα's (not including flexible residues—3, 167–176, and 247) of the uncomplexed and PGA-bound enzyme. The curved arrows show the conformational change of Glu 165 and of the loop composed of residues 167–176. The linear arrow shows the macrodipole moment of helix D_1.

from the open to the closed form. Although the loop has the freedom to move from the open to closed form, all evidence suggests it does so essentially as a rigid body (D. Joseph, M. Karplus, and G. A. Petsko, unpublished results). An analysis of the conformation of the loop in the open and closed forms reveals a number of interesting features. Residues 167 and 168 can be classified as type 3 turns, and residue 170 can be classified as a type 2 turn. Hydrogen bonds involving the carbonyl oxygen of Pro 166 and the amide nitrogen of Ala 169, the carbonyl oxygen of Trp 168 and the hydroxyl of Thr 172, and the amide nitrogen of Leu 174 and the hydroxyl of Thr 172 seem to be responsible for keeping the loop in a single conformation in both the open and closed forms. The interplay between these three intraloop hydrogen bonds and the available dihedral angles of L-amino acids restricts the conformational space of the loop.

An analysis of the relative position of aromatic residues in the bound and unbound enzyme indicates that the loop movement affects interactions among Tyr 164, Trp 168, and Tyr 208. These residues are probably the cause of the changes in the UV spectrum (Jones & Waley, 1979).

A least-squares comparison of all atoms from active site residues Asn 10, Lys 13, His 95, Ser 96, Glu 97, and Glu 165 of the uncomplexed and PGA-complexed structures revealed yet another significant conformational change upon binding. The rms difference for these residues is 0.5 Å, even with a conformational change that places the side chain of the catalytic base, Glu 165, in different positions. In the unbound structure, the carboxylate oxygens of Glu 165 are hydrogen bonded to the backbone amide nitrogen and side-chain hydroxyl group of Ser 96, as well as to the Nε2 of His 95. With PGA bound in the active site, these side-chain hydrogen bonds are broken, and the carboxylate group has moved over 2 Å to interact with the inhibitor.

Discussion

The transition-state theory of catalysis states that active sites accelerate reactions by being structurally and chemically complementary to the transition state of the reaction. Transition-state analogues are useful in enzymology because they can provide clues about the factors that are responsible for catalysis by a particular enzyme. The design of these analogues is based on a mechanistic model for the reaction (Wolfenden, 1969). Structural studies of an analogue complexed to an enzyme can confirm its relevance to the presumed mechanism and reveal interactions that are important for catalysis.

The Origin of Tight Binding of PGA to TIM. PGA binds to TIM at least 100 times more tightly than substrate even though some of the interactions that occur with the true transition state—most notably with the O_1 oxygen—are absent. Our crystallographic results indicate that the origin of this tight binding of PGA for TIM is most certainly due to electrostatic interactions between the inhibitor and enzyme, particularly His 95 and the protonated Glu 165. It is particularly revealing that the K_i is strongly dependent on ionic strength. Binding is strongest at low ionic strength, indicating the importance of electrostatic interactions for this enzyme. It is also interesting to note that binding of PGA to TIM is stronger at pH 5 than at pH 7 (Hartman et al., 1975). This tighter binding at the nonphysiological pH can be attributed to a higher concentration of enzyme protonated at Glu 165.

The importance of electrostatic interactions during the course of catalysis can be traced by analyzing the free energies of binding of various inhibitors. From inhibition studies with inorganic phosphate (K_i = 6 mM for the chicken enzyme) (Burton & Waley, 1968), it can be estimated that the phosphate binding site is responsible for about 3 kcal of the binding energy. By use of the inhibition constant for the substrate analogue glycerol 3-phosphate, 1.4 mM (Nickbarg & Knowles, 1988), as an equilibrium constant between substrate and enzyme, it is estimated that the interactions between the enzyme and the non-phosphate moiety of the substrate are worth about another 1.0 kcal of energy. The binding constant of PGA (K_i = 15 μM) indicates that the charge redistribution that occurs during the course of catalysis (assuming that PGA provides an accurate reflection of the charge configuration at the transition state) is worth at least another 2.5 kcal in energy. Although transition-state analogue inhibitors do not perfectly mimic the transition state, the above analysis indicates they can be used to qualitatively decipher the interactions that are the source of catalysis.

Function of the Conformational Change. A comparison of the PGA-complexed enzyme with the unbound enzyme has revealed essential structural features of catalysis. Figure 3 displays the changes in the active site geometry as TIM goes from the unbound to PGA-bound state. The presence of PGA in the active site has induced the catalytic base, Glu 165, to move over 2 Å, become protonated (not necessarily in this order), and form a hydrogen bond with the carboxylate oxygen of PGA. Although PGA does not exactly mimic the transition state, a similar conformational change must occur when natural substrate is bound. A more dramatic conformational change is the movement of a 10-residue loop that closes over the active site. The same movement has been observed in a low-resolution study of DHAP flowed into TIM crystals (Alber et al., 1981a).

Functional roles for conformational changes in enzymes had been postulated even before any had been observed crystallographically. In the induced-fit mechanism of catalysis, substrate specificity could be enhanced by ligand-induced conformational changes that properly align enzyme groups for

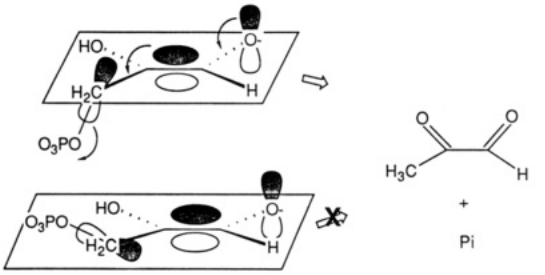

FIGURE 4: Stereoelectronic effects in the TIM-catalyzed reaction. The conformation of the enediolate in the upper left is prone to undergo phosphate elimination. In this conformation, the bridging oxygen sits below the plane of the enediolate intermediate. The sp^3-hybridized orbital of atom C_3 is placed at an orientation that best overlaps with the π-orbital system, a necessary condition for phosphate elimination. The conformation on the lower left has the bridging oxygen planar with the enediolate group. This conformation minimizes the overlap between the sp^3 orbital of C_3 and the π-orbital system. Hence, phosphate elimination is disfavored.

catalysis (Koshland, 1958). It has since been argued that, although the induced-fit theory elegantly describes phenomena associated with allosteric enzymes, it cannot be invoked to explain substrate specificity: ligand-induced conformational changes for *all* substrates lower k_{cat}/K_m by the energy required to distort the enzyme (Fersht, 1985). More recently, it has been proposed that under certain circumstances, such as when the substrate is *totally* surrounded by protein in the active site, conformational changes can increase specificity and catalysis (Herschlag, 1988).

How are the active site geometry and conformational changes that occur in TIM related to catalysis? Clearly, the enzyme has rigidly fixed the factors that are responsible for polarizing the substrate carbonyl oxygens (Belasco & Knowles, 1980). His 95, Lys 12, and the helix composed of residues 95–102, whose macrodipole moment (Hol et al., 1978) induces a positive electric field at its amino-terminal end, hardly move (Figure 3). By polarizing the carbonyl oxygens, TIM has weakened the substrate C–H bonds α to the carbonyls. The carboxylate of Glu 165 then simply swings to position to abstract and transfer a proton from one carbon to another.

The role of the loop movement is a little more difficult to decipher. It has previously been proposed that the role of this loop might be to shield the active site from bulk water (Alber et al., 1987). Indeed, a Connolly surface calculation (Connolly, 1983) using a 1.4-Å probe (which approximates the size of a water molecule) indicates that most of the substrate is shielded from bulk water. As discussed above, electrostatic interactions are thought to be very important during catalysis of the enzyme. Any shielding of bulk water by the loop could effectively decrease the dielectric constant at the active site, thereby increasing the magnitude of electrostatic interactions that occur within it. This interpretation might, at first glance, appear to be contradicted by the ionic strength dependence of PGA binding (Hartman et al., 1975), which indicates that PGA binds strongly at low ionic strength. However, it is not clear whether high ionic strength affects the magnitude of the electrostatic interactions in the active site or simply screens the enzyme's electrostatic gradient, which might function to enhance the association rate of ligands to the active site. It has already been shown that another enzyme operating at the diffusion-controlled limit, superoxide dismutase, enhances the diffusion of substrate to the active site with its electrostatic potential (Sharp et al., 1987). The ionic strength dependence of the superoxide dismutase reaction manifests itself on the association rate of superoxide anion and the enzyme active site (Cudd & Fridovich, 1982; Koppenol, 1981). It is not unreasonable to expect that the same might be true of TIM, although experimental evidence for this is unavailable at this time.

The closure of the loop may also play a role in preventing the phosphate elimination reaction (Figure 1) that occurs so readily with more simple organic bases as catalysts (Richard, 1984). For phosphate elimination to occur, the bridging oxygen must either develop a negative charge or be protonated. The structure indicates there are no functional groups near this oxygen that might either protonate the oxygen or stabilize a developing negative charge. By removing the electrostatic shielding powers of bulk solvent in the active site, the loop serves to discriminate against phosphate elimination by making the formation of an uncompensated negative charge unfavorable.

Prevention of phosphate elimination might also be influenced by stereoelectronic factors (Deslongchamps et al., 1972; Kirby, 1983). In this regard, the orientation of the bridging phosphate oxygen of PGA with respect to the plane of the carboxylate, which mimics the planar enediolate portion of the transition state, has important mechanistic consequences. Stereoelectronic theory predicts that if the in-plane conformation of the O_3 atom observed in the enzyme-bound PGA is also adopted by the intermediate during the enzyme-catalyzed reaction, phosphate elimination would be disfavored. The out-of-plane conformation that would lead to a dihedral angle of 90° for the atoms O1–C1–C2–O3 places the orbitals in the most favored positions for phosphate elimination (Figure 4); the interaction of the loop in its "closed" position with the phosphate group prevents this conformation from being adopted. These observations have led us to predict (Alber et al., 1987) that mutation of the loop would increase the rate of the elimination reaction dramatically. Recently, genetic deletion of four residues from the loop of the chicken enzyme has had such an effect (D. L. Pompliano, A. Peyman, and J. R. Knowles, submitted for publication).

Dynamics and Mechanism of the Conformational Change. The dynamics of loop movement have been studied by computer simulation using chicken TIM coordinates and modeled DHAP (Brown & Kollman, 1987), but the mechanism or trajectory of loop movement was not described. Their study produced a TIM–DHAP structure that is qualitatively similar to some of the details in the structure observed in this study. The most important similarity involves the hydrogen bond made between the loop Gly 171 amide nitrogen and subtrate phosphate oxygen.

The structures of the unbound (preceding paper in this issue) and PGA-complexed TIM may improve the quality of computer simulations by providing more accurate initial and final states of loop movement. Indeed, a recent molecular dynamics study of loop movement addresses the atomic details and forces that trigger the loop to close when substrate is bound to the active site (Joseph et al., unpublished results). In addition, mutagenesis experiments to test hypotheses regarding the loop movement are suggested. These structures and studies are part of a series that we hope will ultimately lead to a detailed understanding of catalysis by this enzyme.

ACKNOWLEDGMENTS

We are very grateful to Richard Wolfenden for first introducing one of us to the TIM–PGA system almost twenty years ago, and for his continued interest in this problem. We thank R. C. Davenport, Diane Joseph, Jeremy Knowles, Elliot Nickbarg, and Dave Pompliano for helpful discussions. We are grateful to Jeremy Knowles for a preprint of his manuscript and to Diane Joseph and Robert Davenport for copies of

manuscripts prior to submission for publication.

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