Segmental Movement: Definition of the Structural Requirements for Loop Closure in Catalysis by Triosephosphate Isomerase†

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ABSTRACT: To determine what drives the closure of the active-site loop in the reaction catalyzed by triosephosphate isomerase, several residues involved in hydrogen bonding between the loop and the bulk of the protein have been altered. It was known from earlier work that the loop serves two functions: to stabilize the reaction intermediate (and the two transition states that flank it) and to prevent the loss of this unstable species into free solution. To discover what elements of the protein are necessary for proper closure of the loop, selective destabilization of the "open" and the "closed" forms of the enzyme with respect to one another has been attempted. The mutant Y164F isomerase has been prepared to evaluate the importance of the structure of the "open" form, and the mutant E129Q, Y208F, and S211A enzymes have allowed investigation of the "closed" form. The integrity of the loop itself has been destabilized by making the T172A isomerase. We have found that only those mutations that destabilize the "closed" form of the enzyme significantly perturb the catalytic properties of the isomerase. The second-order rate constants (k_{cat}/K_m) of the S211A and E129Q enzymes are reduced 30-fold, and that of the mutant Y208F enzyme is reduced 2000-fold, from the level of the wild-type enzyme. The dramatic drop in activity of the Y208F enzyme is accompanied by a 200-fold increase in the dissociation constant of the intermediate analogue phosphoglycolohydroxamate. The most important property of the mobile loop of triosephosphate isomerase lies, therefore, in the stability of the system when the active site contains ligand and the loop is closed.

Proteins are mobile molecules, and the span of motions—in terms both of amplitude and of frequency—is large. These movements range from vibrations at the atomic level on the one hand, to subunit rotations and translations at the quaternary level on the other. Of particular interest to enzymologists is the movement of a defined segment of the molecule, often near the active site of the enzyme. The "induced-fit" proposal of Koshland (1958) was one of the first attempts to explain such conformational changes in proteins as a movement necessary to position catalytic groups and to improve substrate recognition. It is reasonable to presume that these segmental movements may have evolved, for example, to recruit participating amino acid functionalities, to prevent loss of reactive intermediates, to exclude water, or to select a substrate conformation appropriate for reaction (Herschlag, 1988). These mobile segments of protein may be as large as entire domains or as small as short loops. Mobile elements are generally joined to the bulk of the protein by small unstructured sections of polypeptide, or even single peptide units that act as hinges (Huber, 1988).

The most convincing evidence that protein segments or domains move as part of the catalytic process derives from the study of X-ray crystal structures. For example, kinases such as hexokinase (Bennett & Steitz, 1978) and phosphoglycerate kinase (Pickover et al., 1979) appear to ensure transfer of the phospho group from ATP to substrate rather than to water, because the two halves of the protein close down like jaws over the substrates in the ternary complex. In other work, the elegant experiments of Holbrook and co-workers (Clarke et al., 1986) have directly implicated a mobile loop in the reaction catalyzed by lactate dehydrogenase. Mutagenesis of a single conserved arginine residue in this loop abolished polarization of the substrate's carbonyl group, even though loop closure and catalysis could still occur. This work suggested that the binding of anionic pyruvate drives loop closure and brings the arginine side chain of the loop near to the substrate's carbonyl oxygen, in an example of a substrate-induced conformational change. Gerstein and Chothia (1991) have computed that this movement, which is as much as 10 Å, can be described as a classic hinge motion, the loop itself being made of a relatively rigid type II β-turn.

In the present work, we have examined the functioning of another hinged protein: triosephosphate isomerase (TIM).[1] This glycolytic enzyme catalyzes the interconversion of (*R*)-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Scheme I) (Rieder & Rose, 1959; Rose, 1962), and it possesses a mobile loop of about 10 residues, from 166 to 176. This loop has been shown crystallographically to leave the active site open to solvent in the unliganded form of the enzyme and to encapsulate substrate analogs at the active site in the liganded form of the protein (Lolis & Petsko, 1990; Lolis et al., 1990; Noble et al., 1991; Wierenga et al., 1991b). Interestingly, the structure of the loop itself is very similar in the two forms of the enzyme regardless of intermolecular contacts in the crystal (Wierenga et al., 1992), and these residues appear to move as a rigid entity, a kind of "lid" for the active site. Molecular dynamics studies (Joseph et al., 1990; Wierenga et al., 1991a) have confirmed that it is reasonable to view the loop as a polypeptide of defined structure that moves on hinges. The movement of the loop occurs upon substrate binding, and sequesters the reactive intermediate (the enediol, Scheme I) and constrains the substrate into a

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[1] Abbreviations: TIM, triosephosphate isomerase; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PGH, phosphoglycolohydroxamate; GAP, (*R*)-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate.

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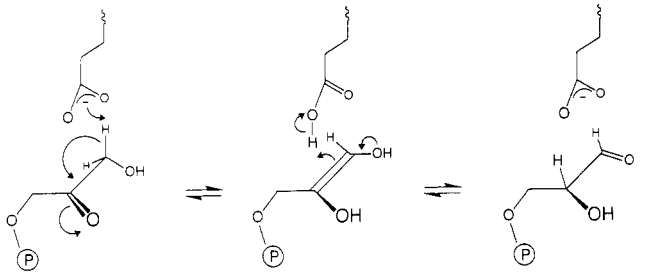
Scheme I: Reaction Catalyzed by Triosephosphate Isomerase[a]



[a] The arrows merely indicate electron reorganization, and do not imply concertedness.

cisoid conformation. In earlier work designed to establish experimentally the functional role of the loop residues, four contiguous amino acids were deleted from the loop by site-directed mutagenesis (Pompliano et al., 1990). The resulting enzyme was a much poorer catalyst (by some 10^5-fold), and it turned out to be incapable of protecting the enediol intermediate from diffusion out into the solvent. Indeed, the intermediate was lost (with consequent elimination of inorganic phosphate to form methylglyoxal) 5 times as often as reprotonation occurred to form the product (DHAP). In the wild-type isomerase, by contrast, the intermediate is lost rarely, less than once in every 10^5 turnovers (Richard, 1991). In addition to preventing the loss of the intermediate into the solvent, the mobile loop of TIM may constrain the enediol in a conformation that is unfavorable for the elimination reaction to occur on the enzyme. Bound substrate is in the fully extended cisoid conformation, and the phosphate ester is held in the same plane as that defined by C-2, C-3, and their two oxygen substituents; this arrangement stereoelectronically disfavors the loss of phosphate by elimination (Kirby, 1983).

The residues of the loop of TIM are highly conserved among the 15 triosephosphate isomerases for which the amino acid sequence is known (Wierenga, 1992; J. E. Adler, personal communication) (Figure 1). Difference electron density maps of liganded and unliganded enzyme (Lolis & Petsko, 1990; Lolis et al., 1990; Noble et al., 1991; Wierenga et al., 1991b) show a movement on the order of 7 Å for the central residue of the loop (threonine-172) upon substrate–analog binding. The lid structure appears to be stabilized by two hydrogen bonds between backbone amide nitrogens and backbone carbonyl groups (170–167, 169–166), and by intraloop hydrogen bonds between the hydroxyl group of threonine-172 and both the backbone amide nitrogen of leucine-174 and the backbone carbonyl of tryptophan-168 (see Figure 2). Furthermore, there are hydrogen-bonding interactions between completely conserved residues of the protein and of the loop that are specific to each form (see Figure 3). Thus, in the "open" structure, the indole nitrogen of tryptophan-168 forms a hydrogen bond to the hydroxyl group of tyrosine-164. Upon loop closure, this bond is broken, and a new one is formed with the carboxyl group of glutamate-129. The loop tryptophan-168 is packed above residues glutamate-165 and proline-166, and on loop closing, tryptophan-168 rotates to lie above the loop. In addition, closure of the loop leads to new hydrogen

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163                                                    178
-Ala-Tyr-Glu-Pro-Val-Trp-Ala-Ile-Gly-Thr-Gly-Leu-Ala-Ala-Thr-Pro-
          2   2   1   1   1   1   1   1   3   4   3   2   2
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FIGURE 1: Amino acid sequence for the mobile loop segment (166–176) in bakers' yeast triosephosphate isomerase (Alber & Kawasaki, 1982). Numbers underneath residues denote the total number of different amino acids at that position in the 15 known sequences of TIM (e.g., 1 indicates that the residue is completely conserved).

bonds between the hydroxyl groups of tyrosine-208 and serine-211 and the amide nitrogens of alanine-176 and glycine-173, respectively. Another interaction is added upon the binding of analog: that between a peripheral phosphate oxygen of the ligand and the backbone NH of glycine-171. Finally, the enzyme's catalytic base, glutamate-165, moves approximately 2 Å upon binding of the ligand.

To determine if the putative "lid" of the isomerase truly acts as an inflexible structural element and to discover what drives loop closure, we have altered several residues involved in the hydrogen-bonding interactions mentioned above. We have determined the kinetic consequences of destabilizing the "closed" form with respect to the "open" form, and we have attempted to destabilize the "open" form with respect to the "closed" form of the enzyme, and to destabilize the loop itself. Additionally, we have explored the utility of using tryptophan-168 as a fluorescence probe of loop movement during catalysis by the enzyme.

EXPERIMENTAL PROCEDURES

Materials. pKK223-3 vectors were obtained from Pharmacia (Piscataway, NJ); M13mp18 phage was obtained from New England Biolabs (Beverly, MA). Oligonucleotide-directed mutagenesis kits were obtained from Amersham (Arlington Heights, IL).

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

Reagents for cell growth, and for purification and assay of the enzymes, were as described by Komives et al. (1991). Native bakers' yeast triosephosphate isomerase was obtained from Sigma Chemical Co. (St. Louis, MO) and further purified on a Mono-Q 10/10 column as described below for the mutant isomerases.

Methods. Rich medium was Luria broth from GIBCO (Grand, Island, NY) supplemented with ampicillin (sodium salt, 200 mg/L) and streptomycin (sulfate salt, 100 mg/L). An extinction coefficient for NADH at 340 nm of 6220 M^{-1} cm^{-1} was assumed (Horecker & Kornberg, 1948). The concentration of purified wild-type yeast triosephosphate isomerase was measured by its absorbance at 280 nm, assuming that $A^{1\%}_{280nm} = 10$ (Norton & Hartman, 1972). Mutant protein assays were conducted by the method of Bradford (1976) using yeast triosephosphate isomerase as the standard. Polyacrylamide stacking gel electrophoresis was conducted according to the method of Laemmli (1970). Protein solutions were concentrated by centrifugation (5000*g*) in Centricon-10 or Centriprep-10 ultrafiltration devices from Amicon (Danvers, MA). PGH was prepared as described by Belasco and Knowles (1980).

The gene encoding triosephosphate isomerase from bakers' yeast was obtained as an insert in M13mp18 phage, and was subcloned into a modified pKK223-3 vector as described by Lodi and Knowles (1991). Site-directed mutations were introduced into the M13 DNA by oligonucleotide-directed mutagenesis. All mutagenesis procedures followed the methods of Eckstein (Nakamaye & Eckstein, 1986). To change threonine-172 to alanine, the mutagenic primer 5′-GCCAAACCGGCACCAATGGC-3′ was used; for threonine-172 to serine, 5′-GCCAAACCGGAACCAATGGC-3′; for tyrosine-208 to phenylalanine, 5′-CGGAACCACCGAATAAGATTC-3′; for serine-211 to alanine, 5′-CCGTTAGCGGCACCACCG-3′; for tyrosine-164 to phenylalanine, 5′-CTGGTTCGAAAGCGACAAGG-3′; for glutamate-129 to glutamine, 5′-CTTTTCTTCCAAAGTTTGACCGATACA-

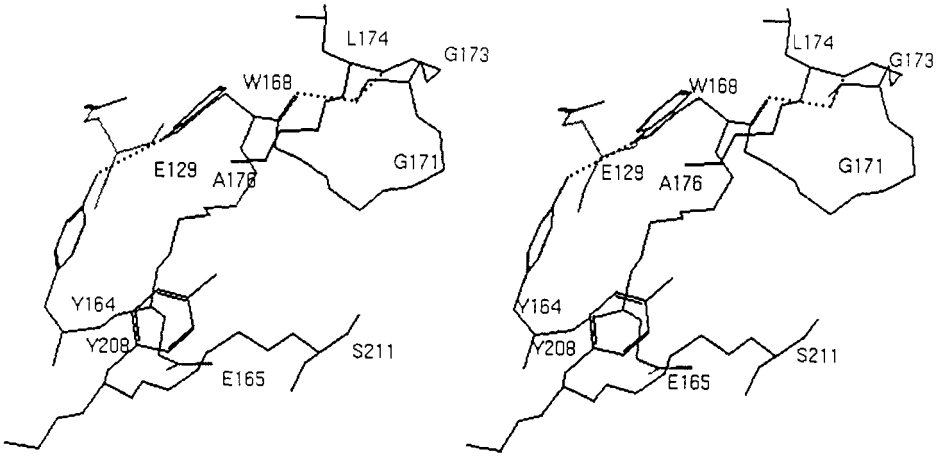

FIGURE 2: "Open" form of the active-site loop of yeast triosephosphate isomerase. Carbonyl oxygens and some side chains have been removed for clarity. Dotted lines denote putative hydrogen bonds. [Taken from Lolis et al. (1990).]

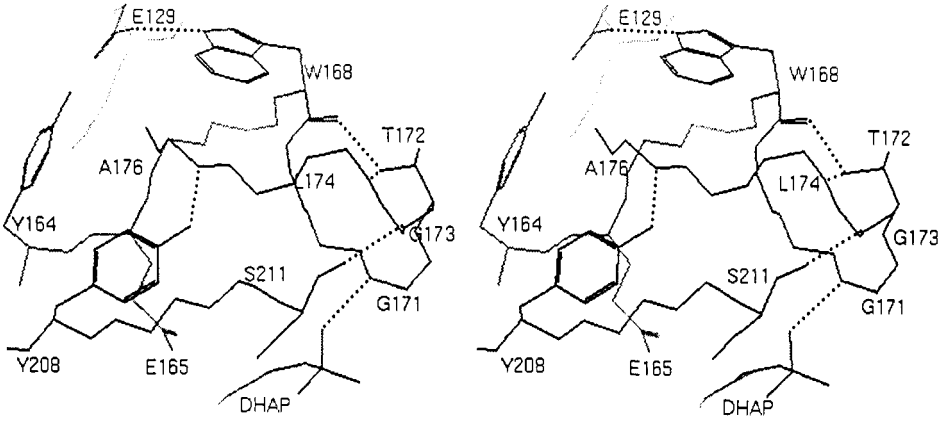

FIGURE 3: "Closed" form of the active-site loop of yeast triosephosphate isomerase with the substrate DHAP modeled onto the bound form of the analog 2-phosphoglycolic acid. Carbonyl oxygens and some side chains have been removed for clarity. Dotted lines denote putative hydrogen bonds. [Taken from Lolis and Petsko (1990).]

CAAGATGAC-3′; for tryptophan-90 to tyrosine, 5′-CCC-AAAATAACATACTTAGC-3′; for tryptophan-157 to phenylalanine, 5′-CGTTAGTAAAGTCCTTAC-3′; and for tryptophan-168 to phenylalanine, 5′-CCAATGGCGAAGAC-TGGTTCG-3′. All mutations were made while the gene remained in M13mp18. The region of interest was sequenced, and the entire gene was then subcloned into the modified pKK223-3 vector (Lodi & Knowles, 1991) and fully sequenced to ensure that no other changes had occurred.

Triosephosphate isomerase activity was measured in 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM). Steady-state kinetic constants (k_{cat} and K_m) were determined as described previously (Nickbarg & Knowles, 1988). One unit of isomerase activity is that amount of enzyme required to convert 1 μmol of (*R*)-glyceraldehyde 3-phosphate to product in 1 min at 30 °C. A value of 26 500 for the isomerase subunit molecular weight was used (Krietsch et al., 1970).

General Procedure for Purification of Yeast Mutant Triosephosphate Isomerases from E. coli DF502(pKK223-3). Cell paste (1 g) of *E. coli* DF502(pKK223-3) obtained from rich medium (100 mL) grown for 15–17 h was suspended in 10 mM Tris-HCl buffer, pH 8.0 (5 mL), and lysed by two passages through a French press at 11 000 psi and 4 °C. Cell debris was removed by centrifugation at 200000*g* for 1 h. All subsequent purification steps were conducted at 4 °C. The supernatant was loaded onto a small column (10 mL) of DEAE-cellulose (DE-52, Whatman) preequilibrated with 10 mM Tris-HCl, pH 8.0. The column was washed with the same buffer (10 mL), and the protein was eluted with 150 mM KCl (30 mL) in 10 mM Tris-HCl buffer, pH 8.0. The eluant was desalted by ultrafiltration. The protein was further purified on a Mono-Q 10/10 column from Pharmacia that had been equilibrated with 10 mM Tris-HCl buffer, 8.0, and eluted with a linear gradient (0–150 mM, 30 mL + 30 mL) of KCl in the same buffer. Fractions were collected and assayed for enzyme activity, and were further analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. If necessary, the protein was purified once more on a Mono Q 10/10 column at pH 7.5 using the conditions described above. Fractions containing the isomerase were pooled, concentrated by ultrafiltration, and desalted into 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM). Typically, 100 mL of culture yielded 1–2 mg of protein that was homogeneous by denaturing gel electrophoresis (Laemmli, 1970).

K_D Determination for Phosphoglycolohydroxamate by Fluorescence Titration. Equilibrium fluorescence measurements were made using a Perkin Elmer LS50 luminescence spectrophotometer at 22 °C. The excitation wavelength was 280 nm (5-nm slit), the emission wavelength was 330 nm (4-nm slit), and a 10-s integration time was used. The decrease in fluorescence at 330 nm upon binding of PGH was monitored. In a typical titration experiment, portions (0.5 μL) of PGH were added to a solution of triosephosphate isomerase (0.4 μM) in 100 mM triethanolamine hydrochloride buffer containing EDTA (10 mM), pH 7.6. PGH was added until no further decrease in fluorescence could be detected. The

Table I: Mutants of Yeast Triosephosphate Isomerase

amino acid	residue no.	replaced by	H-bond removed	H-bond length in "open" form (Å)[b]	H-bond length in "closed" form (Å)[b]
Thr	172	Ala	OH ↔ NH(L174)	3.6	3.6
			OH ↔ CO(W168)	2.6	2.6
Thr	172	Ser	none	3.6	3.6
				2.6	2.6
Tyr	164	Phe	OH ↔ indole(W168)	3.2	5.9
Glu	129	Gln	CO ↔ indole(W168)	3.6	2.4
Tyr	208	Phe	OH ↔ NH(A176)	5.0	2.9
Ser	211	Ala	OH ↔ NH(G173)	10.3	2.7
Gly	171	Pro	NH ↔ *O*-phosphate		2.6
Trp	90	Tyr	*a*		
Trp	157	Phe	*a*		
Trp	168	Phe	indole ↔ OH(Y164)	3.2	5.9
			indole ↔ CO(E129)[a]	3.6	2.4

[a] These mutants were constructed to evaluate the contribution of different tryptophan residues to the observed protein fluorescence. [b] Distances taken from the lengths of the indicated hydrogen bonds in Lolis et al. (1990) and Lolis and Petsko (1990).

observed fluorescence was corrected for dilution of the enzyme and plotted against the total PGH concentration. The data were then fitted to eq 1 using a nonlinear regression program

$$F = F_0 + \{[I]/([I] + K_D)\}(F_\infty - F_0) \quad (1)$$

where F is the observed fluorescence, F_0 is the fluorescence of the unliganded enzyme, F_∞ is the fluorescence of the completely liganded enyzme, [I] is the concentration of added ligand, and K_D is the dissociation constant of the ligand.

RESULTS

Mutant isomerases containing the mutations listed in Table I were expressed and purified, and each purified protein gave a single band upon denaturing polyacrylamide gel electrophoresis. The steady-state parameters for each of these enzymes using (*R*)-glyceraldehyde 3-phosphate as substrate, along with the inhibition constants for the intermediate analog phosphoglycolohydroxamate (PGH), are listed in Table II.

Inhibition constants for PGH were measured under equilibrium conditions from the quenching of the intrinsic protein fluorescence that results from inhibitor binding. The quench data were observed at an emission wavelength of 330 nm upon excitation at 280 nm, these wavelengths being chosen to provide the maximum signal change. These dissociation constants were confirmed by measuring the K_i in a competitive experiment with (*R*)-glyceraldehyde 3-phosphate at a single PGH concentration. Dissociation constants greater than 1 mM could not be measured accurately by equilibrium fluorescence because the high concentration of anionic ligand required to titrate the enzyme interfered with the fluorescence measurements. To demonstrate that fluorescence quenching is a direct indicator of loop environment, three mutant enzymes were constructed: W168F, W157F, and W90Y/W157F. Upon tryptophan excitation at 295 nm, it was found that 65% of the quenchable fluorescence signal may be attributed to tryptophan-168, 35% is derived from tryptophan-90, and tryptophan-157 does not contribute detectably to the fluorescence spectrum of the isomerase. The fluorescence changes reported here are entirely consistent with changes previously observed in the ultraviolet spectra of liganded and unliganded isomerase (Jones & Waley, 1979).

DISCUSSION

The wealth of available structural information about TIM offers an excellent opportunity to study conformational changes during catalysis. The solution of several crystal structures of this enzyme to high resolution has improved our understanding of the active-site loop and of its function. Intrigued by the importance of such a well-defined and conserved element of structure, we have undertaken to identify the necessary components of loop structure and loop closure.

As is evident from inspection of Figures 2 and 3, the side chain of threonine-172 bridges across the loop with hydrogen bonds to the backbone amide nitrogen of leucine-174 and to the backbone carbonyl oxygen of tryptophan-168. In the X-ray crystal structures (Lolis et al., 1990; Lolis & Petsko, 1990), these hydrogen bonds are present in *both* the open and the closed forms, and we expected that if the loop moves as a rigid lid, these hydrogen bonds would contribute to its rigidity. Upon removal of these stabilizing interactions, the ability of the loop to close properly would be diminished, and the catalytic effectiveness of the enzyme would be impaired. In practice, however, neither of the two substitutions, T172A or T172S, affects the catalytic properties of the isomerase or the ability of the enzymes to bind the intermediate analogue PGH (see Table II). Although we cannot deduce the tertiary structure of the mutant isomerases from equilibrium fluorescence measurements, it is evident that there is close similarity between the unliganded and liganded forms of the mutants and those of the wild-type enzyme. Both mutants display the same fluorescence intensities as the wild type in the bound and unbound forms, implying that the loop environment is similar in these three proteins.

In addition to the hydrogen bonds involving threonine-172, there are two additional backbone hydrogen bonds present in the loop in both of its positions. (The isoleucine-170 amide nitrogen is bonded to the valine-167 carbonyl oxygen, and the alanine-169 amide nitrogen is bonded to the proline-166 carbonyl oxygen.) These interactions do not span the lid, but appear to stabilize the structure at the base of the loop segment where it is tethered to the remainder of the protein. Perhaps the loop structure is sufficiently constrained by the fixed position of its ends, so that the new conformations that are accessible to the mutant isomerases do not perturb catalysis or the binding of PGH. Alternatively, a rigid loop may not be necessary for optimal catalysis. In this latter case, the conservation of these interactions across all species so far examined must derive from a selective pressure other than catalytic effectiveness.

In another series of experiments, destabilization of the open structure was attempted by substituting phenylalanine for tyrosine-164, the hydroxyl of which forms a hydrogen bond to the indole nitrogen of tryptophan-168, *only* in the "open" form. If this hydrogen bond is important for stabilization of the "open" form, elimination of this hydrogen bond should produce a protein in which the "open" form of the loop was relatively destabilized. We argued that if a form of the isomerase were constructed the lid of which was always partially closed, transfer of substrate and product into and out of the active site of the enzyme should be hindered. Since the rate-limiting transition state for TIM catalysis involves the release of product GAP from the enzyme, such a change should reduce the catalytic potency of the enzyme. In fact, the catalytic activity of the mutant is barely affected; the second-order rate constant is reduced by a factor of only 2 (Table II). However, tyrosine-164 hydrogen-bonds to a protein residue glutamate-129 in the "closed" form; this hydrogen bond is also disrupted by mutation. That there is no change upon mutation suggests that the destabilization of the "open" form may be balanced by an equal destabilization of the

Table II: Steady-State Kinetic Data for TIM Mutants

mutant	k_{cat}[a] (s^{-1})	K_m[a] (mM)	K_m[b] (mM) (unhydrated)	$(k_{cat}/K_m)_{rel}$[c]	K_i(PGH)[d] (μM)
wild type	8.0 (±0.4) × 10^3	1.3 (±0.1)	0.048 (±0.002)	1.0	16 (±2)
E129Q	5.3 (±0.5) × 10^2	2.6 (±0.5)	0.10 (±0.02)	0.03	43 (±8)
Y164F	6.2 (±0.6) × 10^3	1.9 (±0.3)	0.070 (±0.01)	0.5	6 (±4)
W168F	5.5 (±0.8) × 10^3	1.4 (±0.6)	0.05 (±0.02)	0.6	14 (±2)
T172A	6.8 (±0.6) × 10^3	1.6 (±0.2)	0.059 (±0.007)	0.7	11 (±1)
T172S	8.3 (±0.5) × 10^3	1.5 (±0.1)	0.056 (±0.004)	0.9	13 (±1)
Y208F	7.5 (±0.1)	2.9 (±0.1)	0.107 (±0.004)	0.00042	2800 (±400)[e]
S211A	8.9 (±0.8) × 10^2	4.7 (±0.8)	0.17 (±0.02)	0.031	3.2 (±0.2)
W157F	7.1 (±0.4) × 10^3	1.4 (±0.1)	0.052 (±0.004)	0.8	17 (±4)
W90Y/W157F	8.2 (±0.5) × 10^3	0.9 (±0.1)	0.033 (±0.004)	1.5	9 (±0.5)

[a] With (*R*)-glyceraldehyde 3-phosphate as the substrate. [b] Values for the unhydrated form calculated as in Albery and Knowles (1976). [c] Relative to wild-type isomerase. [d] The K_i for PGH was measured fluorometrically except where noted. [e] The K_i for PGH was measured kinetically with (*R*)-glyceraldehyde 3-phosphate as substrate.

"closed" form. The fluorescence intensity of the unliganded mutant Y164F isomerase is slightly lower than that of wild-type enzyme, but it is not possible to attribute this decrease to a structural rather than an electronic change because of the direct interaction (a hydrogen bond to the primary fluorophore in the wild type) between tyrosine-164 and tryptophan-168. Our efforts to destabilize the open conformation of the enzyme so much that the loop is always closed were not successful. Removal of the hydrogen bond between tyrosine-164 and tryptophan-168 does not slow catalysis, which implies that the conformation of the loop in the "open" structure is not destabilized relative to the "closed" structure. However, rather than disturbing the packing of the loop residues, the effects of which would be more difficult to localize and to define, we have made more conservative mutations, and have removed only single interactions. The highly conserved nature of the hydrophobic loop residues and of the residues of the protein with which they interact indicates, perhaps, that more than hydrogen bonding is important for stabilization of the "open" form and of the loop itself.

In contrast to the structure of the open form, the closed structure has three stabilizing interactions between the loop residues tryptophan-168, glycine-173, and alanine-176 and the protein residues glutamate-129, serine-211, and tyrosine-208, respectively. We have removed each of these interactions separately by mutagenesis. In addition, there is a hydrogen bond between the backbone amide nitrogen of glycine-171 and a peripheral phosphate oxygen of the substrate that is not readily removable. Upon destabilization of the closed form, we expected that loop closure would be less favored and that the enzyme would grip the intermediate less tightly. In fact, each of the three changes, E129Q, Y208F, and S211A, damages the catalytic ability of the isomerase. The second-order rate constants of the E129Q and S211A enzymes are reduced 30-fold, while the binding of PGH is within an order of magnitude of that of wild type. The third mutant isomerase, Y208F, is reduced 2400-fold in activity. This drop is accompanied by a sharp increase in the dissociation constant of PGH. Interestingly, the fluorescence intensity of the unbound S211A enzyme is the same as for wild-type enzyme, but the fluorescence intensity of the bound form is much greater than that of any of the other mutants or of the wild type. The degree of quenching observed is only 12% of the total fluorescence, as compared to 30% that is observed for the wild-type enzyme. This suggests that the structure of the closed lid may be rather different, though confirmation of such a structural change must await X-ray crystallographic investigation. The combination of the diminished catalytic activity of Y208F and the much weaker binding of PGH suggests a loosened grip on the enediol(ate) intermediate by this mutant isomerase, yet the extent to which the binding of PGH precisely parallels that of the enediol(ate) intermediate is unknown. Study of the mutant Y208F isomerase by fluorescence spectroscopy was uninformative because the large dissociation constant required millimolar concentrations of PGH, which interfere with the fluorescence signal.

Our survey of the constituent hydrogen bonds of the active-site loop of TIM has revealed that removal of two intraloop bonds (T172A) or the attempted destabilization of the "open" form (Y164F) does not significantly reduce the catalytic activity of the isomerase. The structural integrity of the loop is not solely dependent on the hydrogen bonding of threonine-172, and may result from the tethering of the ends of the loop to the enzyme or from hydrophobic packing of the conserved loop residues. The stability of the "open" form of the isomerase does not depend on hydrogen bonding to tyrosine-164. This species may be stabilized by hydrophobic interactions. This is in contrast to the "closed" form in which side-chain hydrogen bonds between the loop and at least three different nonloop residues are important for optimal catalysis. We conclude that the most significant aspect of catalysis involving the mobile loop of triosephosphate isomerase lies in *the stabilization of the "closed" form*. Further study of the Y208F mutant, to determine whether it is the *process* by which the loop closes or the precise *structure* of the liganded form that slows catalysis, is detailed in the following paper (Sampson & Knowles, 1992).

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