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Stabilization of a Reaction Intermediate as a Catalytic Device: Definition of the Functional Role of the Flexible Loop in Triosephosphate Isomerase†

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ABSTRACT: The function of the mobile loop of triosephosphate isomerase has been investigated by deleting four contiguous residues from the part of this loop that interacts directly with the bound substrate. From the crystal structure of the wild-type enzyme, it appears that this excision will not significantly alter the conformation of the rest of the main chain of the protein. The specific catalytic activity of the purified mutant enzyme is nearly 10^5-fold lower than that of the wild type. Kinetic measurements and isotopic partitioning studies show that the decrease in activity is due to much higher activation barriers for the enolization of enzyme-bound substrate. Although the substrates bind somewhat more weakly to the mutant enzyme than to the wild type, the intermediate analogue phosphoglycolohydroxamate binds much less well (by 200-fold) to the mutant. It seems that the deleted residues of the loop contribute critically to the stabilization of the enediol phosphate intermediate. Consistent with this view, the mutant enzyme can no longer prevent the loss of the enediol phosphate from the active site and its rapid decomposition to methylglyoxal and inorganic phosphate. Indeed, when glyceraldehyde 3-phosphate is the substrate, the enediol phosphate intermediate is lost (and decomposes) 5.5 times faster than it reprotonates to form the product dihydroxyacetone phosphate. Triosephosphate isomerase has evidently evolved its mobile loop to bind tightly the highly reactive intermediate enediol phosphate, in a conformation that disfavors the wasteful elimination process. These studies support the increasing recognition of the essential function (as distinct from the mere existence) of mobile loops near the active sites of enzymes.

Loops pervade the proteins of known structure (Leszcynski & Rose, 1986; Richardson, 1981). Considered to be nonregular elements of protein secondary structure, loops are contiguous segments of polypeptide, variable in size and sequence, that trace a curved path through space. Loops may exist in a range of conformations and have a relatively small end-to-end distance separating the segment termini. The side chains of loop residues are usually hydrophilic and are almost always situated on the protein surface where they are well placed to interact with ligands such as substrates, hormones, and effectors.

The question of whether loops have a functional role in ligand recognition or in enzyme catalysis is not one that has often received direct experimental support, despite the existence

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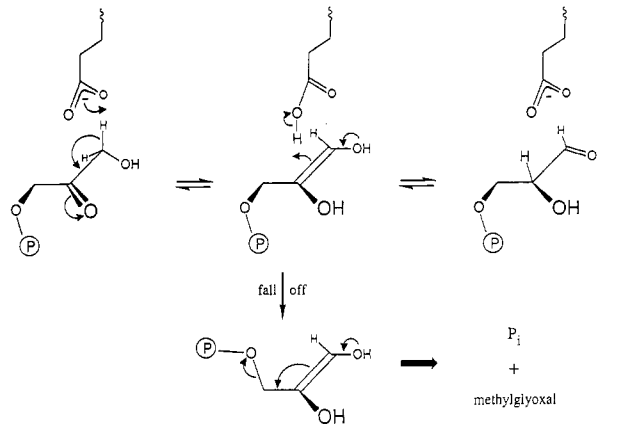
of many persuasive arguments. One of the first such expressions was the "induced-fit" proposal of Koshland (1958), which endowed enzymes with the capacity to wrap around their substrates, both recruiting functional groups to locations appropriate for the subsequent catalytic events and refining the act of substrate recognition. [Though in respect of the latter, it has been pointed out that enzyme specificity is only improved if the mobile residues completely surround the substrate on binding and provide more intrinsic binding energy with the correct substrate compared to an improper substrate (Herschlag, 1988).] Strictly, almost any conformation change that is triggered by substrate binding, whether it involves the movement of a protein domain, of a loop of residues, or of a single amino acid side chain, can be classified as induced fit. For example, there is crystallographic evidence that indicates that protein domains move to encapsulate the bound substrates of hexokinase (Bennett & Steitz, 1978) and of phosphoglycerate kinase (Pickover et al., 1979). In these cases, it has been reasonably suggested that the domain closure has the useful effect of sequestering the substrates so that the appropriate acceptor group (rather than a water molecule) is phosphorylated by ATP. Analogously, the flexible C-terminal tail of phosphoglycerate mutase may act as a lid that closes over the substrate during the catalytic act (Winn et al., 1981), and the nucleotide binding loop of adenylate kinase that is seen in different locations when the structures of the two major crystal forms are compared may regulate the access of ATP to its binding site (Fry et al., 1986; Egner et al., 1987). In addition, the movement of peptide loops in carboxypeptidase A (Ludwig et al., 1967) and in lactate dehydrogenase (Adams et al., 1973; Clarke et al., 1986) appears from crystallographic studies to bring new functional groups into position for the catalytic reaction. Yet the mere existence of such conformation changes does not demand that they play a role in catalysis, and the link between substrate-induced structural alterations and the functional consequences of these changes has not often been made. Loop deletion and loop swap experiments have demonstrated the importance of certain "Ω" loops for the electron transport mediated by cytochrome *c* (Fetrow et al., 1989). In another example, the removal of five amino acids from a surface loop of phospholipase A_2 was shown both to enhance the enzyme's catalytic activity and to alter its substrate specificity (Kuipers et al., 1989). In the present paper, we examine the catalytic consequences of a loop closure that occurs in triosephosphate isomerase when substrates or inhibitors bind at the active site.

Triosephosphate isomerase, the glycolytic enzyme that catalyzes the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Scheme I) (Rieder & Rose, 1959; Rose, 1962), possesses an intriguing flexible loop that appears to close the active site when substrate binds, by interacting with the peripheral phosphate oxygen atoms of the ligand in the active site. X-ray crystallographic studies (Banner et al., 1976; Alber et al., 1981) show that residues 168–177 of the triosephosphate isomerases from both chicken muscle and yeast leave the active site open to solvent in the native enzymes but close down upon the substrate dihydroxyacetone phosphate or the competitive inhibitors phosphoglycolate, phosphoglycolohydroxamate, or (even) inorganic phosphate when these materials bind to the enzyme. The phosphate group of the bound substrate lies near to the mouth of the active site, while the triose part of the substrate fills the active-site pocket and interacts with the presumed catalytic groups of Lys-13, His-95, and Glu-165. Bound substrate is in the fully extended cisoid conformation, with the

Scheme I: Reaction Catalyzed by Triosephosphate Isomerase



phosphate ester in the same plane as the carbonyl group of dihydroxyacetone phosphate. Difference electron density maps of unliganded enzyme and liganded enzyme suggest a substantial movement (on the order of 10 Å) of the loop residues toward the active site when substrate binds (Alber et al., 1981). This movement causes the main-chain NH groups of residues Gly-171 and (possibly) Gly-173 to interact with the peripheral phosphate oxygens of the bound ligand (Alber et al., 1981). In so doing, the loop residues appear to close the active site and to form specific donor hydrogen bonds that bind the substrate's phosphate group. Such an interaction is consistent with the absolute specificity of the enzyme for phosphorylated substrates [even the isosteric but only singly charged analogue dihydroxyacetone sulfate does not bind detectably to the enzyme (Belasco et al., 1978)] and satisfies the obvious requirement for some enzyme groups to help to neutralize the charges on the dianionic phosphate ester.

The amino acid sequence of the flexible loop is highly conserved among the 12 triosephosphate isomerases for which sequence data are available (Nickbarg, 1988). This fact, together with the knowledge that triosephosphate isomerase is an extremely effective catalyst (Knowles & Albery, 1977), underscores the importance of the flexible loop for the proper functioning of the enzyme. To be an efficient catalyst, the enzyme must both accelerate the desired transformation and suppress any side reactions that would detract from the formation of product. The isomerization of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, whether uncatalyzed or enzyme catalyzed, proceeds through an enediol phosphate intermediate (Scheme I) (Rieder & Rose, 1959; Rose, 1962; Richard, 1984). This intermediate is very short-lived in solution and rapidly suffers elimination to yield P_i and the enol of methylglyoxal (Richard, 1984). Stereoelectronic considerations (Deslongschamps et al., 1972; Kirby, 1983) suggest that elimination from the enediol phosphate is least favored if the orbital overlap between the enediol π system and the C–O bond that links the phosphate group to C-3 is minimized. If the enzyme were to constrain the substrate in the fully extended conformation, which certainly seems assured when the flexible loop is in place, the unwanted phosphate elimination side reaction would be minimized (Alber et al., 1981). In this way, the loop could be responsible for the more efficient transformation of substrate into product.

To probe the role of the loop in the reaction catalyzed by triosephosphate isomerase, we have used site-directed mutagenesis to delete four contiguous amino acid residues from this flexible segment (Chart I). Examination of the X-ray structure for the yeast isomerase (Alber et al., 1981) showed that the Cα–Cα distance between Ala-169 and Lys-174 is

Chart I: Loop Residues of Triosephosphate Isomerase[a]

168 177
- Pro - Val - Trp - Ala - ***Ile*** - ***Gly*** - ***Thr*** - ***Gly*** - Lys - Thr - Ala - Thr - Pro - Gln -

[a] The ten residues of the mobile loop are indicated by the bracket, and the four deleted residues are shown in bold type.

about 5 Å. Since the normal Cα–Cα distance in an extended amino acid chain is 3.8 Å, we reasoned that the protein backbone would not be significantly distorted if the intervening four residues from 170 to 173 were excised and a new peptide bond were introduced between Ala-169 and Lys-174. The four deleted residues include the two that are particularly implicated in binding the substrate's phosphate group, and the resulting shortened loop would be unable to complete the act of substrate binding. We report here the purification and kinetic characterization of this mutant isomerase. Removal of the portion of the flexible loop that interacts with the substrate's phosphate group has severe and illuminating consequences for the reaction catalyzed by triosephosphate isomerase.

Experimental Procedures

Materials. Escherichia coli strain DF502, a streptomycin-resistant variant that lacks the endogenous bacterial isomerase, was a gift from D. Fraenkel. Native chicken muscle triosephosphate isomerase was purified according to Putman et al. (1972). Phosphoglycerate kinase (yeast), pyruvate kinase (rabbit muscle), triosephosphate isomerase (rabbit muscle), glyoxalase I (yeast), alkaline phosphatase (calf intestine), glycerophosphate dehydrogenase (rabbit muscle), and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were obtained as ammonium sulfate suspensions from Boehringer Mannheim Biochemicals (Indianapolis, IN). Traces of contaminating triosephosphate isomerase activity were removed from the dehydrogenases by treatment with bromohydroxyacetone phosphate as described by Plaut and Knowles (1972).

(*RS*)-Glyceraldehyde 3-phosphate (diethyl acetal, monobarium salt), dihydroxyacetone phosphate (lithium salt), NAD^+, NADH, Dowex 50W (H^+ form, 100–200 mesh, 4% cross-linked), and QAE-Sephadex A-50 (Cl^- form) were from Sigma Chemical Co. (St. Louis, MO). Ammonium sulfate was of special enzyme grade from Schwarz/Mann (Cambridge, MA). Bromohydroxyacetone phosphate was prepared by R. T. Raines according to the method of de la Mare et al. (1972). Phosphoglycolohydroxamate [bis(cyclohexylammonium) salt] was synthesized by J. G. Belasco as described in Belasco and Knowles (1980). [1(*R*)-^{3}H]Dihydroxyacetone phosphate (9 Ci/mol) was prepared by R. T. Raines according to the method of Herlihy et al. (1976). Phospho[1-^{14}C]-glycerate (14.1 Ci/mol) was prepared according to the method of Guilford et al. (1987). [1-^{14}C]Glyceraldehyde 3-phosphate and [1-^{14}C]dihydroxyacetone phosphate were prepared enzymatically from 3-phospho[1-^{14}C]glycerate as described by Nickbarg et al. (1988). Tritiated water (4.5 Ci/mL) was from Amersham (Chicago, IL). Scintiverse II scintillation cocktail was from Fisher (Medford, MA). Flo-Scint III scintillation cocktail was obtained from Radiomatic Instruments and Chemical Co. (Tampa, FL). Bactotryptone and Bacto yeast extract were from Difco Labs (Detroit, MI). Triethylamine was distilled before use. All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

A synthetic 42-residue oligonucleotide complementary to bases 487–507 (codons 163–169) and bases 520–540 (codons 174–180) of the wild-type chicken triosephosphate isomerase gene and designed to delete the intervening bases 508–519 (codons 170–173) was synthesized by the phosphoramidite method on a MilliGen Biosearch 7500 DNA synthesizer. All DNA restriction enzymes and T4 ligase were from New England Biolabs (Beverly, MA). Sequencing primers for the chicken triosephosphate isomerase gene were synthesized on the DNA synthesizer.

Methods. Rich medium was LB (Miller, 1972) supplemented with ampicillin (sodium salt, 200 mg/L). All media were prepared in deionized, distilled water. Samples (5–1000 μL) for radiochemical analysis were dissolved in scintillation cocktail (4–6 mL) and counted in a Beckman LS1801 automatic liquid scintillation counter. Scintillation counting for both ^{3}H and ^{14}C was done after calibration with Beckman counting standards and by use of the double-label counting programs supplied with the instrument. pH was measured with a Corning 245 pH meter fitted with a Sigma E5634 electrode and calibrated at room temperature with Fisher standard buffers. Ultraviolet and visible absorbances were measured on a Hewlett-Packard 8452A diode array spectrophotometer thermostated with a Brinkmann RMS 6 temperature controller.

Mixtures of radiolabeled 3-phospho-D-glycerate, *sn*-glycerol 3-phosphate, and methylglyoxal were separated on a Pharmacia Mono-Q HR 5/5 column fitted into a Waters HPLC system consisting of a Model 680 gradient controller, two Model 510 pumps, and a U6K injector. The separation involved an isocratic wash (4 min) with 10 mM triethylammonium formate buffer, pH 3.8, followed by a linear gradient (10–1000 mM, over 20 min) of triethylammonium formate buffer, at a flow rate of 2 mL/min. Column eluent was mixed on-line with scintillation fluid and monitored with a flow-through scintillation counter (Flo-One/Beta, Model CT, from Radiomatic Instruments and Chemical Co.). Fractions were collected and counted in the Beckman scintillation counter.

All enzymatic reactions were carried out at 30 °C, unless noted otherwise. Inorganic phosphate content was determined by the method of Ames (1966). Methylglyoxal was quantitated by end-point assay using glyoxalase I as described by Racker (1957). An extinction coefficient for NADH at 340 nm of 6220 M^{-1} cm^{-1} was assumed (Horecker & Kornberg, 1948). The concentration of purified chicken triosephosphate isomerase was measured by its absorbance at 280 nm, assuming that $A_{280}^{1\%} = 1.21$ (Miller & Waley, 1971). Triosephosphate isomerase was assayed at pH 7.6 by the method of Plaut and Knowles (1972). A subunit M_r of 26 500 was assumed (Putman et al., 1972) and kinetic parameters quoted are for a single subunit. Polyacrylamide stacking gel electrophoresis was conducted with 12.5% (w/v) acrylamide and 0.33% (w/v) bis(acrylamide) gels in the presence of 0.1% (w/v) sodium dodecyl sulfate according to the method of Laemmli (1970). The stacking gel was buffered with 0.5 M Tris-HCl buffer, pH 6.8, and the separating gel was buffered with 1.5 M Tris-HCl buffer, pH 8.8. The gels were fixed in 10% (w/v) trichloroacetic acid and then stained for protein with Coomassie Blue. The molecular weight standards (Bethesda Research Labs, Bethesda, MD) were lysozyme (14 300), β-lactoglobulin (18 400), carbonic anhydrase (29 000), ovalbumin (43 000), bovine serum albumin (68 000), phosphorylase *b* (97 400), and myosin H-chain (200 000). Protein concentrations were determined by using the Bradford (1976) method with bovine serum albumin as standard. Protein solutions were concentrated by ultrafiltration with Amicon PM-10 membranes or by centrifugation in an Amicon Centricon 10 ultrafiltration apparatus. Dialysis tubing

Table I: Purification of the Loop Mutant Triosephosphate Isomerase

step	total protein (g)	total units	sp catalytic act. (units/g)	x-fold purification	yield (%)
crude lysate	10.2	158	15	1.0	100
ammonium sulfate precipitation	3.46	98	25	1.7	62
QAE chromatography	1.29	46	36	2.3	29

(Spectropor, M_r 12000–14000 cutoff) was pretreated according to Brewer (1974).

Mutagenesis. Oligonucleotide-directed mutagenesis of the purified virion M13mp10.TIM DNA was done by using the primer described above according to the Eckstein protocol (Taylor et al., 1985a,b; Nakamaya & Eckstein, 1986; Sayers et al., 1988) and by using materials from Amersham Corp. (Arlington Heights, IL). Competent *E. coli* TG1 cells were transformed with the mutagenized DNA. Phage single-strand DNA was purified from six random clones. Each clone was sequenced with modified T4 DNA polymerase by the Sanger chain termination method (Sanger et al., 1977; Sequenase DNA sequencing kit, United States Biochemical Corp., Cleveland, OH). Phage RF DNA was purified from clones with the desired mutant DNA sequence, and a *Pst*I-*Sac*I fragment containing the mutant triosephosphate isomerase gene was isolated. This fragment was subcloned into the high-expression phagemid pBSX1c (Hermes et al., 1989), producing the phagemid pBSX1c.tdl. The phagemid pBSX1c.tdl was used to transform TG1, and purified single-strand DNA isolated from a subclone was sequenced. One subclone, designated ptΔ1, was used to transform *E. coli* DF502.

Steady-State Kinetics. The steady-state parameters K_m and k_{cat} were determined by a nonlinear least-squares fit of the data using the Marquardt (1963) algorithm. The concentration of a solution of phosphoglycolohydroxamate was determined by colorimetric assay (Ames, 1966) of the inorganic phosphate released by alkaline phosphatase. Mutant isomerase was assayed at six different concentrations of glyceraldehyde 3-phosphate in the presence of varying concentrations of phosphoglycolohydroxamate. The dissociation constant for the enzyme–inhibitor complex, K_i, was also calculated from a nonlinear least-squares fit of the data. Isotopic labeling studies (both the "transfer" and "discrimination" experiments) were carried out as described by Nickbarg et al. (1988).

Purification of the Mutant Isomerase. All glassware was either new or acid-washed to eliminate the possibility of contamination of triosephosphate isomerase from other sources. *E. coli* DF502(ptΔ1) cell paste (81 g, wet weight) was obtained from 20 L of culture grown for 40 h at 37 °C with vigorous shaking. The cells were harvested by centrifugation (13000*g*) in a Sorvall continuous flow rotor at 4 °C. All subsequent purification steps were performed at 4 °C. The cell paste was resuspended in 1 L of 10 mM Tris-HCl buffer, pH 7.6, and crude lysates were prepared by passing the cell suspension twice through a Manton-Gaulin mill at 9000 psi. Cell debris was removed by centrifugation (15000*g*, 30 min). Finely ground ammonium sulfate (516 g) was added slowly to the stirring supernatant (1325 mL). The suspension was then gently stirred for 2 h before the precipitated protein was removed by centrifugation (16000*g*, 30 min). More ammonium sulfate (328 g) was added to the supernatant (1500 mL), the solution was allowed to stir for 2 h, and the precipitated protein was collected by centrifugation. The protein pellet was dissolved in 150 mL of 10 mM Tris-HCl buffer, pH 7.6, and the solution dialyzed exhaustively against the same buffer. The dialyzed solution was loaded onto a column (25 cm × 20 cm^2) of QAE-Sephadex A-50 that had been equilibrated with 10 mM Tris-HCl buffer, pH 7.6. The column was washed with the same buffer, and the mutant isomerase was eluted with a linear gradient of KCl (0–300 mM, 1.2 L + 1.2 L) in the same buffer. Fractions (20 mL) were collected and assayed for enzymatic activity and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Fractions containing pure mutant isomerase were pooled and then concentrated by ultrafiltration.

Table II: Kinetic Parameters for the Loop Mutant Triosephosphate Isomerase

kinetic parameter[a]	wild-type[b] enzyme	mutant enzyme	ratio (wild type/ mutant)
$\vec{k}_{cat}$ (s^{-1})	430	0.0081	53000
$\vec{K}_m$ (mM)	0.97	8.2	0.12
$\overleftarrow{k}_{cat}$ (s^{-1})	4300	0.045	95000
$\overleftarrow{K}_m$ (mM)	0.47	2.3	0.20
K_{eq}	21	19	1.1
K_i (mM) (for arsenate)	11	12	0.90
K_i (μM) (for phosphoglycolohydroxamate)	7.1	1500	0.005

[a] The parameters $\vec{k}_{cat}$ and $\vec{K}_m$ refer to dihydroxyacetone phosphate as substrate; $\overleftarrow{k}_{cat}$ and $\overleftarrow{K}_m$ refer to glyceraldehyde 3-phosphate as substrate. Reaction rates, in each direction, were determined from the time-dependent appearance of product. [b] Values for the wild-type enzyme are from Putman et al. (1972).

Results

Mutant isomerase was purified according to Raines et al. (1986), as summarized in Table I. The purified protein gave a single band on polyacrylamide gel electrophoresis under denaturing conditions, which indicated a molecular weight of the mutant enzyme indistinguishable from that of the wild type. The steady-state kinetic parameters for the mutant enzyme, along with the inhibition constants for the intermediate analogue phosphoglycolohydroxamate and for inorganic arsenate, are listed in Table II. Bromohydroxyacetone phosphate inactivates the mutant isomerase much more slowly than the wild-type enzyme.

The extent of tritium transfer from substrate [1(*R*)-^{3}H,1-^{14}C]dihydroxyacetone phosphate to the ultimate product 3-phosphoglycerate (where s_0 is the specific radioactivity of the substrate at the start of the reaction and p is the specific radioactivity of the product) catalyzed by the mutant isomerase was less than 1%, even after the reaction was 80% complete. Determination of the relative specific radioactivity of both the remaining substrate and the product was made from the ^{3}H/^{14}C ratio of the fractions from the HPLC column. The results for the relative specific radioactivity of the remaining substrate (s/s_0) at various extents of reaction are shown in Figure 1.

With [1-^{14}C]dihydroxyacetone phosphate as substrate, product labeling experiments for the mutant isomerase were performed in tritiated water at six extents of reaction. The specific radioactivity of tritium p, in the product glyceraldehyde 3-phosphate, which was converted in situ to phosphoglycerate (expressed as a fraction of the solvent specific radioactivity, x), was determined at various extents of reaction (Table III). Labeling experiments were also run the opposite direction,

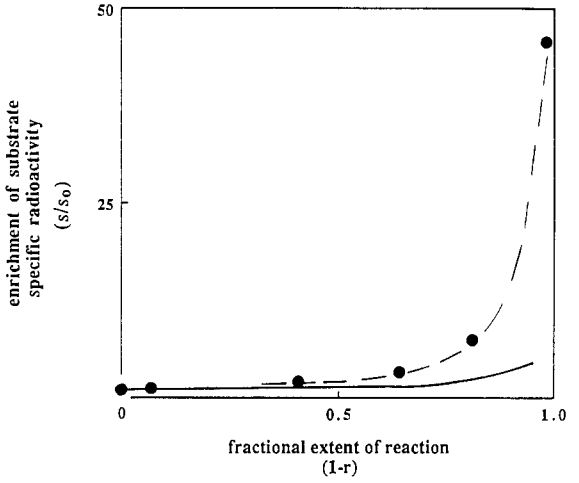


FIGURE 1: Variation in the specific radioactivity of the remaining substrate [1(*R*)-^{3}H]dihydroxyacetone phosphate with the extent of reaction. The solid line illustrates, for comparison, the behavior of the wild-type enzyme (Herlihy et al., 1976).

Table III: Incorporation of Solvent Tritium into Triose Phosphate Product during the Reaction Catalyzed by the Mutant Enzyme

glyceraldehyde 3-phosphate as substrate		dihydroxyacetone phosphate as substrate	
$(1 - r)^a$	$(s/x)^b$	$(1 - r)^a$	$(p/x)^b$
0.23	0.11	0.03	0.12
0.36	0.11	0.16	0.12
0.64	0.12	0.46	0.12
0.93	0.084	0.79	0.14
0.99	0.11		

[a] Fractional extent of reaction. [b] Specific radioactivity of the product relative to that of the solvent.

using [1-^{14}C]glyceraldehyde 3-phosphate as substrate. The values for the tritium content of the ultimate product glycerol phosphate, s (expressed as a fraction of x), at several extents of reaction are tabulated in Table III.

Since one of the suggested functions of the intact loop is to bind the substrate in a conformation that disfavors the elimination of phosphate from the enediol phosphate intermediate, we checked the stability of the triose phosphates in the presence of the mutant enzyme. Incubation of mutant isomerase with substrate under reversible conditions (that is, in the absence of coupling enzymes or their cofactors) resulted in rapid decomposition of the triose phosphates (Figure 2). Control reactions containing no enzyme, or containing an equal concentration of wild-type enzyme, showed virtually no loss of triose phosphates (<5%) over the same time period. The probable cause of the rapid decomposition of substrate observed with the mutant enzyme is by phosphate elimination from the enediol phosphate intermediate to produce methylglyoxal and inorganic phosphate. This expectation was confirmed by assay of methylglyoxal (using glyoxalase I and glutathione), which demonstrated the time-dependent and isomerase-dependent production of the elimination products.

To determine how rapidly the mutant isomerase produces methylglyoxal, the enzyme reaction was run under irreversible conditions (that is, in the presence of coupling dehydrogenase and the appropriate cofactor) in each direction. Quantitation of triose phosphates remaining, inorganic phosphate released, and methylglyoxal formed showed reasonable agreement in molar terms. The partition ratio for the formation of methylglyoxal versus dihydroxyacetone phosphate, when glyceraldehyde 3-phosphate is the substrate, was 5.5 in favor of methylglyoxal. When the reaction was run in the other direction, with dihydroxyacetone phosphate as substrate, the ratio of methylglyoxal to product was 0.65. When glyceraldehyde 3-phosphate is the substrate, therefore, the mutant protein catalyzes the decomposition to methylglyoxal more than 5 times faster than it catalyzes the conversion to dihydroxyacetone phosphate.

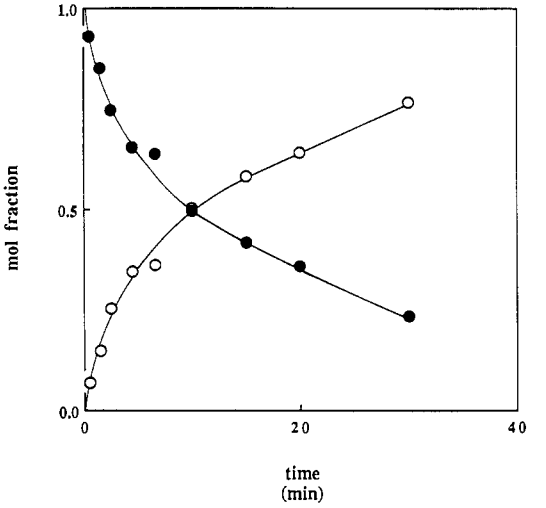


FIGURE 2: Disappearance of total triose phosphates (closed circles) and appearance of methylglyoxal (open circles) in the presence of the loop deletion mutant isomerase as a function of time. [1-^{14}C]-Glyceraldehyde 3-phosphate (8.3 mM, 1.70×10^{-4} μCi/μmol) was incubated with mutant isomerase (1 mg/mL) in triethanolamine hydrochloride (pH 7.6)–10 mM EDTA buffer at 30 °C. Portions were quenched at timed intervals. The triose phosphates and methylglyoxal were separated from the reaction mixture by HPLC and their relative concentrations determined.

In the absence of a crystal structure of the mutant enzyme, an energy-minimized structure was generated on the basis of the crystallographic coordinates for the wild-type yeast enzyme. The coordinates for yeast triosephosphate isomerase containing bound phosphoglycolohydroxamate were altered so as to remove the four deleted amino acids (residues 170–173), and the connectivity of the atoms was changed so as to introduce a peptide bond between residues 169 and 174. The altered coordinate set was submitted to CHARMM (Brooks et al., 1984) for energy minimization. The wild-type coordinates were minimized analogously, and the resulting structures were displayed using the molecular graphics software QUANTA (Figure 3). The energy minimization for the mutant enzyme left the coordinates virtually unchanged: the root mean square difference was 0.17 Å for all Cα backbone atoms excluding those for the deleted residues, 170–173. However, a dramatic difference in the degree of protection of the active site from solvent was evident for the loop deletion mutant. This enzyme is missing four key residues, and it displays a gaping hole that can no longer prevent free access of small molecules to the active site. The substrate's phosphate group is exposed, and the substrate as a whole is less tightly and less rigidly bound by the mutant enzyme since it lacks at least one hydrogen bond (and probably two) from the main chain of the loop of the native enzyme to the bound substrate.

DISCUSSION

Triosephosphate isomerase catalyzes a simple chemical reaction that involves the transfer of protons, to and from C-1 and C-2 and to and from the carbonyl and hydroxyl group oxygens, of its triose phosphate substrates. The pressures of evolution have selected for an enzyme that accelerates the enolization steps of the isomerization reaction to the point where these are not rate limiting, and the diffusion of gly-

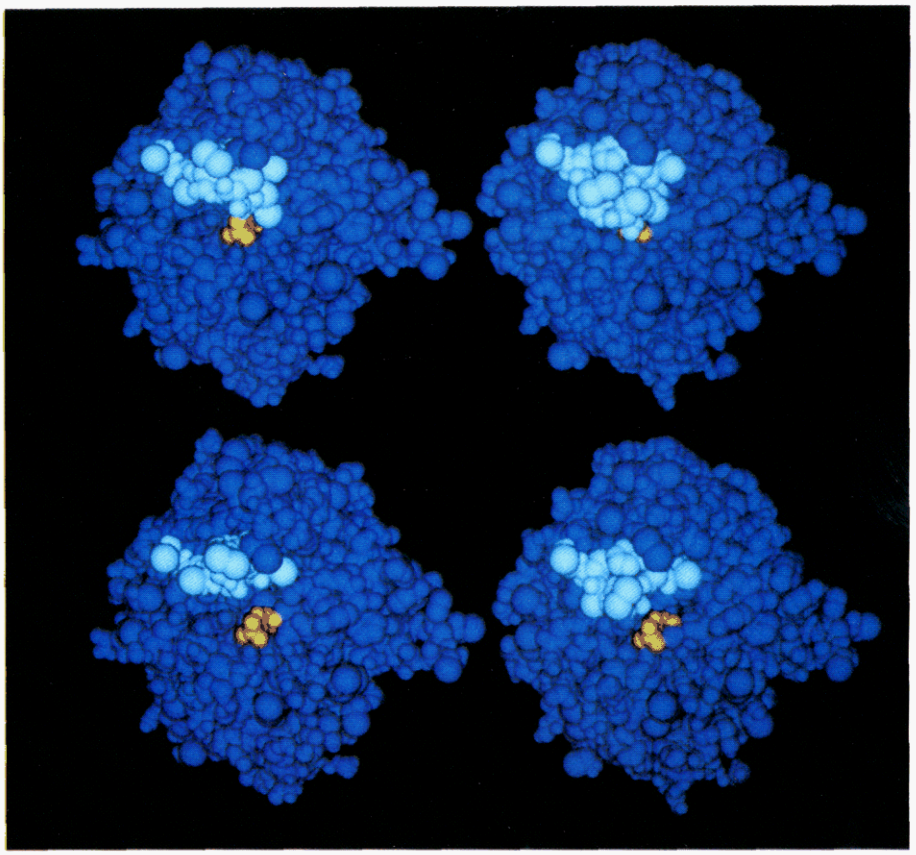


FIGURE 3: Computer-generated views of the active-site region of the wild-type and mutant triosephosphate isomerases. The atoms of the loop region (residues 168–177) are light blue, the atoms of the bound inhibitor phosphoglycolohydroxamate are orange, and the atoms of the rest of the protein are dark blue. Upper left: wild-type isomerase in the open loop form, unliganded state (the inhibitor is shown bound, for visual reference only) [coordinates from Davenport (1986)]. Upper right: wild-type isomerase in the liganded state with the inhibitor bound [coordinates from Davenport (1986)]. Lower left: mutant isomerase in the open loop form (the inhibitor is shown bound, for visual reference only) (energy-minimized structure, based on the amended coordinates from the structure above). Lower right: mutant isomerase in the closed loop form (energy-minimized structure, based on the amended coordinates from the structure above).

ceraldehyde 3-phosphate in and out of the active site limits the turnover rate of the enzyme. Indeed, compared to a simple basic catalyst such as acetate ion, the enzyme speeds up the interconversion of its substrates by some 10 orders of magnitude (Richard, 1984).

One intriguing feature of the enzyme is the loop that extends from residue 168 to residue 177. When substrate binds to the enzyme, the residues of this loop, which are relatively disordered in the crystal structure of the unliganded enzyme, fold down over the substrate and become highly ordered. Although the loop is near the active site, it does not appear to contain any residues that are directly involved in the chemistry of the enolization steps, and one may ask whether it serves an essential catalytic function. Many enzymes contain mobile segments, and it has often been suggested that such flexible loops represent an element of induced-fit mechanisms. A clear example of the induced-fit mechanism is the reaction catalyzed by lactate dehydrogenase. Binding of substrate to this enzyme induces the movement of a polypeptide loop that closes over the active site of the enzyme, bringing Arg-109 (a residue that is conserved among all lactate dehydrogenases) into the active site. Site-directed mutagenesis of this residue (to Gln) has suggested that it is responsible for polarizing the carbonyl group of the substrate and for stabilizing the transition state for pyruvate reduction (Clarke et al., 1986). Favorable interactions between the enzyme and the transition state thus appear to be gained by the movement of this loop. In the case of triosephosphate isomerase, the mobile loop seems also to clamp down upon the bound substrate, and while this closure does not involve the recruitment of any new catalytic groups, the substrate does become sequestered from solvent and is, presumably, more tightly bound by virtue of the new hydrogen bonds that are made from one or two glycyl NH groups to the substrate's phosphate oxygens.

The amino acid sequence of the mobile loop of triosephosphate isomerase (Chart I) is also suggestive. In many nucleotide-binding proteins, a common loop, which connects a β-sheet with an α-helix and contains the consensus sequence G-X-G-X-X-G(A), is found in the region of (pyro)phosphate binding (Möller & Amons, 1985; Wierenga et al., 1986). The glycine residues in these loops allow for a relatively sharp bend between the β-sheet and the α-helix, and alterations to these residues have deleterious effects on binding or catalysis by a variety of enzymes. For example, mutation of the middle glycine residue of the consensus loop sequence to any other amino acid (except proline) in the *ras*-gene-encoded protein (p21) results in oncogenic activation (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982; Seeburg et al., 1984). Crystallographic studies have indicated that the GTP-binding loop of the transforming *ras* protein is enlarged compared to the normal protein (Tong et al., 1989), and such a change in the phosphate-binding pocket could explain the reduced GTPase activity of the mutant. Mutations in the G-X-G-X-X-G motif for other proteins have also been described. Thus in the nucleotide-binding loop of adenylate kinase, replacement of the middle glycine (Gly-10) by valine led to a 20-fold decrease in the K_m for ATP (Reinstein et al., 1988). The fact, therefore, that the flexible loop of triosephosphate isomerase contains the G-X-G-X-X-A (Chart I) sequence and binds the substrate's phosphate group is curious and makes one ask

whether the isomerase has capitalized on this motif in order to bind its substrates and intermediate more specifically.

Examination of the crystal structure of chicken triosephosphate isomerase revealed a splendid opportunity to test the importance of the flexible loop. Residues 170–173, which form a looplet within the larger flexible segment, contain that portion of the loop which interacts directly with the bound substrate. At least one, and possibly two, of these residues, Gly-171 and Gly-173, form main-chain hydrogen bonds with the anionic oxygen atoms of the substrate's phosphate group. Since the Cα–Cα distance between the flanking looplet residues (about 5 Å, between residues 169 and 174) is close to the normal peptide Cα–Cα distance (3.8 Å), we supposed that a peptide bond could be introduced between these two residues without significantly distorting the overall structure. Four codons (170–173) were therefore deleted by site-directed mutagenesis, and the mutant protein was expressed at high levels in *E. coli*. After purification of the mutant protein to homogeneity, the steady-state kinetic parameters were determined (see Table II). Not unexpectedly, we observed a precipitous drop, on the order of 10^4–10^5-fold, in the values of k_{cat} for each substrate. In contrast, the K_m values increased by only 5–8-fold, suggesting that substrate binding to the enzyme is not seriously impaired. The value of K_i for the intermediate analogue phosphoglycolohydroxamate was, however, increased by a factor of 200.

To pinpoint the source of the catalytic impairment, more detailed kinetic experiments were necessary. Through a series of experiments that trace the fates of either a substrate-derived or a solvent-derived tritium label, information about the internal energetics for the reaction catalyzed by triosephosphate isomerase can be deduced. These experiments have been discussed earlier, both quantitatively (Albery & Knowles, 1976) and qualitatively (Raines et al., 1986; Nickbarg et al., 1988). If [1(R)-^{3}H,1-^{14}C]dihydroxyacetone phosphate is substrate, the tritium label may be found either in the product glyceraldehyde 3-phosphate (through a "transfer" of label mediated by the enzymic base), in the solvent (due to label exchange with solvent after proton abstraction), or in the substrate that remains. The extent to which the tritium label is transferred from substrate [1(R)-^{3}H,1-^{14}C]dihydroxyacetone phosphate to product is 0.5–1.1%, depending on the extent of reaction, compared to the 3–6% transfer seen with the reaction catalyzed by the wild-type chicken muscle isomerase. From the same experiment, the tritium content of the remaining substrate can be determined. The specific radioactivity (of ^{3}H) is related to the primary tritium isotope effect on proton abstraction (which results in the preferential consumption of unlabeled substrate molecules) and is inversely related to the extent of back-reaction from the enediol phosphate intermediate (since most of these molecules' label will have been washed out into solvent). With [1(R)-^{3}H,1-^{14}C]dihydroxyacetone phosphate as substrate, the remaining substrate becomes enriched in tritium by 8.5-fold after 80% of reaction, an indication that the primary tritium isotope effect dominates these processes. When the reaction catalyzed by the mutant isomerase is run in tritiated solvent with unlabeled dihydroxyacetone phosphate as substrate, an 8.3-fold discrimination against tritium is seen in the product, and when glyceraldehyde 3-phosphate is substrate, the discrimination against tritium in the product is 9.1-fold. The size of these isotope effects and the dramatic decrease in the values of k_{cat} for the mutant enzyme indicate that the activation barriers for the two enolization steps have been increased very significantly.

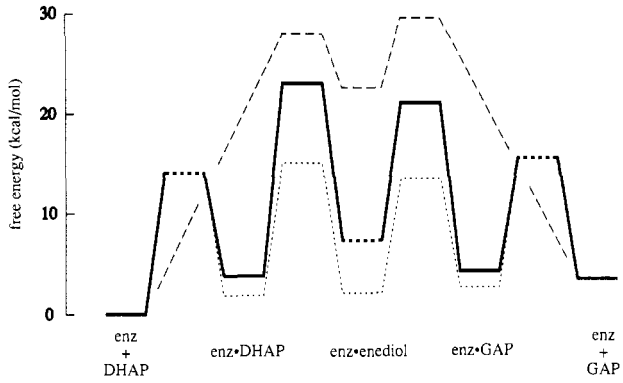


FIGURE 4: Free energy profiles for the reaction catalyzed by wild-type isomerase (dotted line), a general-base catalyst of pK_a 7 (dashed line), and mutant isomerase (heavy solid line). The wild-type enzyme's profile is from Albery and Knowles (1977), and the profile for the general-base catalyst is calculated from the data of Richard (1984). The mutant enzyme's profile was derived by using the value of k_{cat} with dihydroxyacetone phosphate as substrate (corrected for the rate of enediol phosphate decomposition to methylglyoxal) and the relative rates of enediol phosphate collapse to methylglyoxal and to triose phosphate product. The relative free energy of bound enediol phosphate was calculated from the value of K_i of phosphoglycolohydroxamate for the mutant enzyme relative to that for the wild-type enzyme. The heavy dashed lines indicate free energy levels that are only estimated.

The results discussed above, taken together, allow a rough free energy profile to be constructed for the mutant isomerase. This is illustrated in Figure 4. The removal of the four loop residues has altered the energetics of the reaction so that it begins to resemble that for catalysis by a nonenzymic base of pK_a 7, a primordial catalyst for this transformation (Richard, 1984). The rates of substrate enolization to form the enediol phosphate intermediate are dramatically slowed by the deletion of four amino acid residues from the loop, and the mutant enzyme fails to stabilize the enediol phosphate intermediate (using the binding of the intermediate analogue phosphoglycolohydroxamate as a crude measure of the binding of the enediol phosphate). Indeed, it would appear from Figure 4 that *the mutant enzyme has lost its grip on the enediol phosphate intermediate*. Since in free solution the enediol phosphate intermediate eliminates P_i to give methylglyoxal at a rate that is at least 100 times faster than reprotonation (Richard, 1984), an efficient isomerase cannot afford to bind this intermediate weakly. Indeed, the wild-type enzyme loses the intermediate very infrequently compared to the rate of overall substrate turnover, and the transformation of substrate to product is extremely efficient. Quite the opposite is true for the mutant enzyme that has a truncated loop. Incubation of either of the triose phosphates with mutant enzyme leads to substrate decomposition (Figure 2). Methylglyoxal and inorganic phosphate are formed, and the loopless mutant enzyme is actually rather better at catalyzing the elimination reaction than it is at catalyzing the isomerization reaction! When glyceraldehyde 3-phosphate is substrate, the enediol phosphate intermediate partitions in a ratio of 5.5 to 1 in favor of the elimination products. That is, for every six enediol phosphate intermediates that are formed by the enolization of glyceraldehyde 3-phosphate, five fall off the enzyme and decompose, and only one is reprotonated to form dihydroxyacetone phosphate. In the reverse direction with dihydroxyacetone phosphate as substrate, the ratio of methylglyoxal to glyceraldehyde 3-phosphate is 0.65. Alber et al. (1987) suggested earlier that methylglyoxal synthase might be a "loopless" triosephosphate isomerase, and it now seems clear that this could be true. One function of the loop in triose-

phosphate isomerase is thus to prevent the elimination side reaction. Whether the loop prevents release of the enediol phosphate into solution, or whether the loop simply locks the phosphate group of the substrate in a conformation from which elimination is stereochemically disfavored while still on the enzyme, is not yet clear.

Even if the efficiency of the mutant enzyme were not compromised by the wasteful liberation of the unstable reaction intermediate, the free energy profile of Figure 4 emphasizes the kinetic importance to the enzyme of binding the enediol phosphate. Starting either with the nonenzymic reaction or with the reaction catalyzed by the loopless mutant (Figure 4), catalytic power is gained by specifically binding the reaction intermediate. By lowering the relative free energy of the bound enediol phosphate intermediate, the enzyme would accelerate the overall reaction by reducing the relative free energies of the two transition states that flank it and (though less so, by the application of Hammond's postulate) of the complexes of the enzyme with dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The calculated structure of the mutant enzyme containing a mimic of the enediol phosphate intermediate (phosphoglycolohydroxamate) at the active site offers some insight into the molecular basis for the kinetic results we have observed. The inhibitor's phosphate group is significantly more exposed in the mutant enzyme compared to the wild-type enzyme (Figure 3). The truncated loop can no longer wrap around the substrates and the intermediate to enhance their binding. A comparison of the hydrogen bonds in the region of the active site for the wild-type enzyme and the mutant enzyme shows that at least one hydrogen bond (from NH of Gly-171) to a peripheral oxygen of the substrate's phosphate group is present in the wild-type enzyme but is unavailable in the mutant. There also appears to be a hydrogen bond in the wild-type isomerase between the main-chain NH of Gly-173 and Oγ of Ser-210 that is missing and uncompensated for in the mutant protein. This interaction may aid in binding the phosphate group by helping to anchor the loop residues to the body of the protein. While more detailed discussion must await crystallographic information on the liganded mutant enzyme, it is clear that triosephosphate isomerase has exploited a proven structural motif to position and bind the enediol phosphate intermediate. Alteration of this motif produces a mutant enzyme that can no longer bind the reaction intermediate appropriately. Proton abstraction processes become rate limiting, and the enzyme loses hold of its intermediate. Catalysis is slow, and the throughput efficiency of the enzyme is compromised.

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Kinetic Competence of Enzymic Intermediates: Fact or Fiction?†

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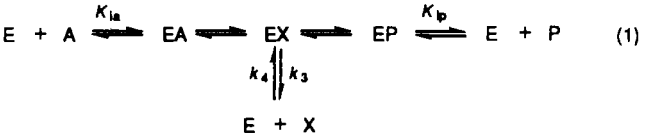
ABSTRACT: A number of enzymatic reactions involve intermediates that are not normally released during the reaction. Whether such an intermediate when added to the enzyme reacts as fast or faster than the normal substrates, and thus is "kinetically competent", depends on the degree to which the equilibrium constant for forming the intermediate from the substrates is different on the enzyme surface and in solution, as well as on the relative affinities of the enzyme for substrate and intermediate. Similar values for these equilibrium constants require that the intermediate react slowly, while a far more favorable value for intermediate formation on the enzyme allows the intermediate to react at up to the diffusion-limiting rate. When one intermediate is formed from two substrates, it may react much more rapidly than when two intermediates are formed from two substrates, or one from one. Comparison of the kinetics of the putative intermediate(s) and the substrate(s) can reveal a great deal about the mechanism of the catalytic reaction and the kinetic barrier that normally keeps the intermediate(s) on the enzyme.

Many enzyme-catalyzed reactions involve multiple steps and intermediates that do not dissociate from the enzyme during the reaction. Such putative intermediates are in some cases stable enough to be prepared and tested as alternate substrates, and the question then arises how fast should such molecules react? One view is that in order for the intermediate to be "kinetically competent" it must react as fast or faster than the overall reaction of the substrate or substrates that it replaces. An alternative view is that because the "off" rate constant for an intermediate that does not normally dissociate is very small, the "on" rate constant must also be small, or the intermediate will be so tightly bound that it would exist in a deep hole in the free-energy profile for the reaction and its reaction in either direction would be too slow. Which view is correct?

It turns out that both situations are possible, with the controlling factor being the ratio of equilibrium constants between substrate and intermediate on and off the enzyme. Consider the simple mechanism



(1)

where A and P are the normal substrate and product and X is the intermediate. K_{ia} and K_{ip} are the dissociation constants of A from EA and P from EP. X does not normally dissociate

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