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Active site of triosephosphate isomerase: *In vitro* mutagenesis and characterization of an altered enzyme

(glycolytic enzymes/site-directed mutagenesis)

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ABSTRACT **We have replaced the glutamic acid-165 at the active site of chicken triosephosphate isomerase with an aspartic acid residue using site-directed mutagenesis. Expression of the mutant protein in a strain of *Escherichia coli* that lacks the bacterial isomerase results in a complementation phenotype that is intermediate between strains that have no isomerase and strains that produce either the wild-type chicken enzyme or the native *E. coli* isomerase. The value of $\vec{k}_{cat}$ for the purified mutant enzyme when glyceraldehyde 3-phosphate is the substrate is 1/1500th that of the wild-type enzyme, and the $\vec{K}_m$ is decreased by a factor of 3.6. With dihydroxyacetone phosphate as substrate, the $\overleftarrow{k}_{cat}$ value is 1/240th that of the wild-type enzyme, and $\overleftarrow{K}_m$ is 2 times higher. The value of K_i for a competitive inhibitor, phosphoglycolate, is the same for the mutant and wild-type enzymes, at 2×10^{-5} M. By treating the enzyme-catalyzed isomerization as a simple three step process and assuming that substrate binding is diffusion limited, it is evident that the mutation of glutamic acid-165 to aspartic acid principally affects the free energy of the transition state(s) for the catalytic reaction itself.**

Triosephosphate isomerase (TIM; D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1) is a glycolytic enzyme that interconverts dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) (Fig. 1). The second-order rate constant for the reaction in the downhill direction (GAP → DHAP) is $\approx 10^{10}$ times faster for TIM catalysis than for acetate ion catalysis, and it is close to the theoretical limit for the diffusive encounter of substrate and enzyme (1, 2).

Kinetic and mechanistic analyses of the TIM-catalyzed reaction have led to a plausible pathway for this transformation (Fig. 1). There is good evidence that the isomerization proceeds through a *cis*-enediol (or enediolate) intermediate (3). B^- in Fig. 1 represents a base, almost certainly the carboxylate group of glutamic acid-165, which abstracts the pro-*R* hydrogen from substrate DHAP. The conjugate acid of this group (BH in Fig. 1) subsequently protonates the enediol(ate) intermediate to produce GAP. The assignment of this role to glutamic acid-165 rests on chemical modification experiments (4–6) and on high resolution crystallographic data (7). An electrophilic residue (HA in Fig. 1) is thought to stabilize the developing negative charges on the oxygens at C-1 and C-2 during formation of the enediol(ate), polarizing the carbonyl group in the enzyme–substrate complex and facilitating the C-H bond cleavage reactions (7–9). From measurements of the fate of isotopic labels in the substrate and in solvent, Albery and Knowles were able to determine the rate constants for each step of the reaction and to construct a Gibbs free energy profile for the catalyzed process (10).

Site-directed mutagenesis, combined with crystallographic and kinetic studies, provides a method for confirming the catalytic mechanism of an enzyme and for delineating the contribution of particular interactions to catalysis and to binding. Studies of this type have recently been initiated on tyrosyl-tRNA synthetase (11, 12), β-lactamase (13), dihydrofolate reductase (14), aspartate carbamoyltransferase (15), and yeast TIM (16). In the present case, we have chosen not to alter the chemical nature of an essential enzymatic catalytic group, but merely to move it. We have used a simple mutagenesis protocol to incorporate a synthetic oligonucleotide encoding aspartic acid in place of glutamic acid-165 into a gapped plasmid. Our results confirm that glutamic acid-165 is critical for the proton shuttling processes and suggest that this residue makes only a small contribution to the binding of the reaction intermediates.

METHODS

Strains, Media and DNA. Strains have been described (17). Rich medium is YT medium (18) supplemented with 0.1% dextrose. Minimal medium is M63 medium (18), with carbon sources at a concentration of 0.2%. Plasmids were prepared by either alkaline or boiling methods (19–21). The heptadecanucleotide was synthesized by the phosphotriester method (22).

Enzymes. Calf intestinal phosphatase was obtained from Boehringer Mannheim; restriction enzymes, T4 DNA ligase, and DNA polymerase (Klenow fragment) were from New England Biolabs; and T4 polynucleotide kinase was from New England Nuclear.

Mutagenesis. The hybrid *trp–lac* promoter of plasmid pX1 directs the expression in *Escherichia coli* of chicken TIM, which is encoded on a 1046-base-pair (bp) *Nco* I fragment (17). Plasmid pX1 was modified by incorporating a heptadecamer coding for aspartate at amino acid position 165, where there is normally a glutamate residue. Fig. 2 illustrates the method for hybridizing the oligomer to gapped plasmids. The technique is a modification of that of A. Gautier (personal communication). Plasmid pX1 (0.1 pmol), that had been cut with *Pvu* II at a unique site outside of the insert, was mixed with *Nco* I-cut pKK233-2 (0.1 pmol) and the kinased synthetic oligomer (2 pmol) in a final volume of 10 μl of 50 mM Tris buffer, pH 7.5, containing $MgCl_2$ (10 mM) and 2-mercaptoethanol (7 mM). After incubation for 3 min in a boiling water bath, the solution was rapidly chilled in an ice bath, and the reaction mixture was brought to 50 mM Tris buffer, pH 8.0/10 mM $MgCl_2$/20 mM dithiothreitol/1 mM rATP/0.625 mM dATP/0.625 mM dTTP/0.625 mM dCTP/0.625 mM dGTP/DNA polymerase I Klenow frag-

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Abbreviations: TIM, triosephosphate isomerase; bp, base pair(s); DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate.

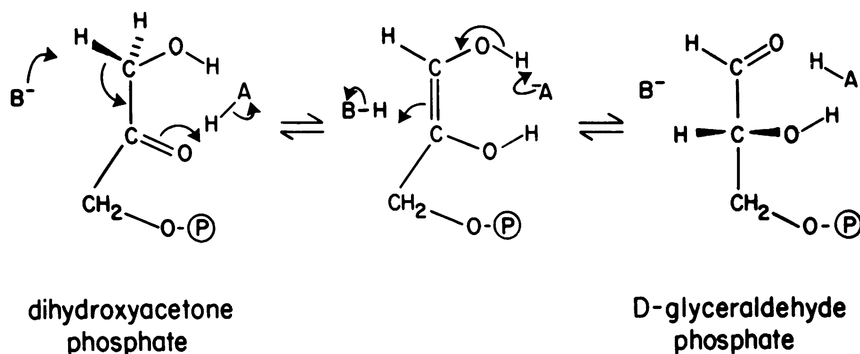


FIG. 1. Reaction catalyzed by TIM. B^- represents glutamic acid-165 and HA is an electrophilic residue(s), possibly lysine-13 and/or histidine-95, that stabilizes the developing negative charge on the substrate carbonyl oxygen during formation of the intermediate. Although we have depicted the enediol, it is possible that the actual intermediate is the enediolate.

ment (0.125 unit/μl)/T4 DNA ligase (10 units/μl), in a total vol of 40 μl. This polymerization/ligation reaction was left at 15°C for 12 hr. Aqueous NaOAc (500 μl; 0.3 M) containing EDTA (10 mM) was then added, and the DNA was precipitated with ethanol, dried, and dissolved in 10 mM Tris buffer (40 μl), pH 8.0/1 mM EDTA. *E. coli* strain DH1 was then transformed with 20 μl of this DNA solution (23).

The 20,000 transformants that grew on a rich plate containing ampicillin were screened using the ^{32}P-labeled heptadecanucleotide (24). Seven positive colonies were purified by twice restreaking and rescreening with the same probe. Plasmid DNA (2 ng) from an alkaline minipreparation (20) was then used to transform competent DF502 cells. The resulting transformants were tested for growth on selective carbon sources. The sequence of the coding region of ptm2 was determined by the chemical method (25).

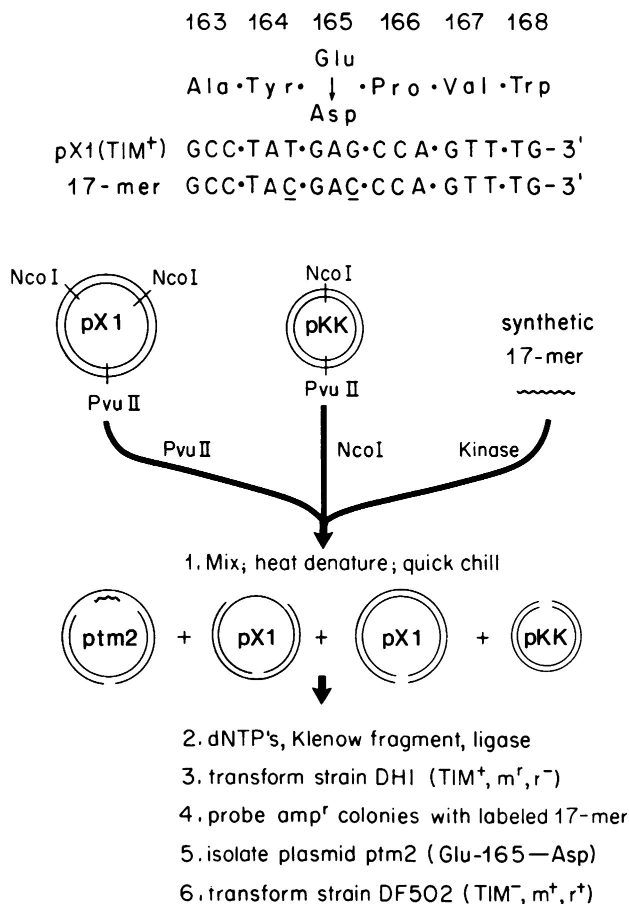


FIG. 2. Method for isolating the glutamic acid-165 → aspartic acid mutation. Plasmid pX1 contains the TIM coding sequence on a 1046-bp *Nco* I fragment. The parental vector (lacking this insert) is pKK233-2, which is labeled pKK in the figure.

Purification of the Mutant Enzyme. About 35 g (wet weight) of DF502/ptm2 was harvested from 15 liters of culture grown to 4 OD_{550} units in shaker flasks in rich medium containing ampicillin (100 μg/ml) and streptomycin (100 μg/ml). The cell cake was stored at −70°C. Great care was taken during all purification steps to avoid any contamination from the wild-type isomerase by using new or acid-washed glassware and disposable plastic pipettes and tubes. After thawing and resuspending in H_2O (70 ml), bacteria were lysed by 3 passes through a French pressure cell (American Instrument) at 14,000 psi. The lysate was spun at 165,000 × g for 15 min to remove cell debris and the NADH oxidase activity, which causes high background in assays of the crude lysate. Solid $(NH_4)_2SO_4$ (Bethesda Research Laboratories, enzyme grade) (218.0 g) was dissolved in H_2O (394 ml) at room temperature and mixed with the supernatant (106 ml). This mixture was stirred at 4°C for 24 hr, and then spun for 2 hr at 10,800 × g. To the supernatant (540 ml), finely ground $(NH_4)_2SO_4$ (92.3 g) was added slowly with stirring. After stirring the suspension for 24 hr at 4°C, the precipitate was collected by centrifugation at 10,800 × g for 5 hr. Resuspension of the pellet in H_2O (5 ml) yielded a solution (9 ml) that was dialyzed against 20 mM Tris·HCl (2 × 2 liters), pH 7.20/0.2 mM EDTA. Samples from the crude lysate and from the $(NH_4)_2SO_4$ cuts were also dialyzed before being assayed. The dialyzed enzyme solution (12.2 ml) was centrifuged at 12,000 × g for 20 min and then passed through a Pharmacia pre-filter. This material (11.5 ml) was loaded onto a column (25 cm × 20 cm^2) of QAE Sephadex A-50 (Pharmacia), which had been prepared according to the manufacturer's specifications and equilibrated with 10 mM Tris·HCl (pH 7.3). The column was eluted with the same buffer, and fractions (18 ml) were collected at a rate of ≈110 ml/hr. Fractions were assayed for enzyme activity, and samples of the peak fractions were further analyzed by NaDodSO_4/polyacrylamide electrophoresis on a 10% gel (26), after which protein was visualized with Kodavue stain (Eastman Kodak). The fractions that contained the mutant isomerase (fractions 32–44) were pooled and concentrated by dialysis against 20 mM Tris·HCl, pH 7.3/1 mM EDTA/polyethylene glycol [M_r, >20,000; 10% (wt/vol)] at 4°C for 48 hr. The final volume after dialysis was 1 ml, and the protein concentration was 0.46 mg/ml.

Assays. TIM activity was assayed as described (17, 27), except that the final assay volume was 1 ml and EDTA was not included in the reaction mixture. The protein concentration was measured using the Bradford Coomassie protein assay (28). A standard curve was constructed using various concentrations of purified chicken TIM.

RESULTS

Mutagenesis. Plasmid pX1 contains the coding region of chicken TIM under transcriptional control of an upstream hybrid *trp–lac* promoter and a ribosome binding site. The plasmid was made by inserting a 1046-bp *Nco* I fragment carrying the TIM sequence (the initial methionine codon forms part of one of the *Nco* I sites) into the unique *Nco* I site of vector pKK233-2 (17). As Fig. 2 shows, we generated the mutant by creating a single-stranded gap covering the TIM sequence, hybridizing with the synthetic oligonucleotide, filling in the gaps, and then ligating the plasmid together. Plasmid pX1, digested with *Pvu* II, which cuts outside the TIM insert, was mixed with linearized (*Nco* I) pKK233-2 (the parental vector: pKK in Fig. 2) and with the synthetic 17-mer that differed from the wild-type sequence at two positions. After heat denaturation and chilling the mixture on ice, DNA polymerase I (Klenow fragment) and DNA ligase were added to fill in and to seal the gaps. Strain DH1 was then transformed with this DNA and the resulting ampicillin-resistant transformants were screened with the radioactive 17-mer end-labeled with ^{32}P. Sequence analysis of one of the seven resulting positive clones, ptm2, revealed that the oligonucleotide had been incorporated at the correct site and that the rest of the TIM sequence was unchanged.

Complementation. *E. coli* DF502 cannot produce a functional isomerase because of a deletion in the chromosomal *TIM* gene (29, 30). The strain cannot grow on plates with either lactate or glycerol as the sole carbon source, but it does grow on plates containing both: presumably, this is because lactate can be metabolized to GAP and glycerol can be metabolized to DHAP. When DF502 is transformed with pX1, however, the transformants grow on either lactate or glycerol; thus, chicken TIM can satisfactorily replace the bacterial enzyme in *E. coli* (17). When plasmid ptm2 was used to transform DF502 and transformants were selected for ampicillin resistance, it was found that the transformed cells grow on lactate but not on glycerol. Glycerol selection may be more stringent than lactate selection because of an accumulation of the toxic intermediate of glycerol metabolism, methylglyoxal, in cells with a less than optimum complement of TIM (31).

Purification. We obtained 0.46 mg of purified mutant TIM from 35 g (wet weight) of bacteria that contained 4.35 g of soluble protein. The yield for the 850-fold purification was 11%. NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 3) shows that the protein is homogeneous. We also purified the wild-type chicken enzyme from strain DF502 containing plasmid pX1. The specific catalytic activities of purified mutant TIM, of purified wild-type chicken TIM made in *E. coli*, and of purified chicken TIM are 6.4 units/mg, 9000 units/mg and 11,000 units/mg, respectively.

Enzymatic Properties. The values of k_{cat} and K_m for both DHAP ($\vec{k}_{cat}$, $\vec{K}_m$) and GAP ($\overleftarrow{k}_{cat}$, $\overleftarrow{K}_m$) as substrate were determined by analyzing the data with the aid of the program HYPERO (32). Since the reaction mixture contains arsenate when DHAP is substrate (causing the irreversible removal of the GAP product), the apparent $\vec{K}_m$ for DHAP must be corrected for arsenate inhibition. We found that the value of K_i for arsenate is $26 \pm 3 \times 10^{-3}$ M for the mutant enzyme, somewhat greater than the value of $13 \pm 1 \times 10^{-3}$ M that we observed for the native chicken enzyme. Table 1 compares the values of k_{cat} and K_m for the mutant TIM and the native chicken enzyme. With DHAP as substrate, $\vec{k}_{cat}$ for the mutant enzyme is lower by a factor of 240 and $\vec{K}_m$ is ≈2 times the value for the wild-type enzyme. With GAP as substrate, $\overleftarrow{k}_{cat}$ is 1500 times lower, and $\overleftarrow{K}_m$ is smaller by a factor of 3.6 for the mutant enzyme. Because the values of $\vec{K}_m$ and $\overleftarrow{K}_m$ differ for mutant and wild-type TIM, we can be sure that the activity we are observing is not due to contaminating wild-type enzyme. The Haldane equation, $K_{eq} = (\overleftarrow{k}_{cat}/\overleftarrow{K}_m)(\vec{K}_m/\vec{k}_{cat})$, yields a K_{eq} of 21 ± 3, compared to the value of 20.8 obtained earlier (27). Both triose phosphates exist in solution as mixtures of hydrated and unhydrated species, only the latter of which are substrates for TIM. The constants with subscripts "unhyd" in Table 1 relate to the reaction of the true (unhydrated) substrates (10, 33, 34).

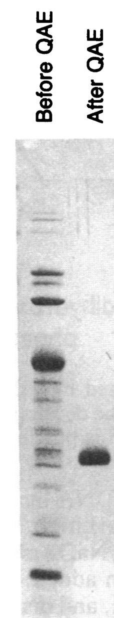


FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoretic analysis of the purification of the mutant enzyme. Eight micrograms of protein from the resuspended 95% $(NH_4)_2SO_4$ pellet and 1 μg of the pooled, concentrated, and dialyzed TIM-containing fractions from the QAE column were electrophoresed on a 10% gel (26). The specific catalytic activities of these samples were 0.14 and 6.4 units per mg of protein, respectively.

Wolfenden has argued that phosphoglycolate, which is a potent competitive inhibitor of TIM, binds to the enzyme analogously to the *cis*-enediol(ate) intermediate (35). To evaluate the effect of our mutation on the binding of this intermediate, we compared the values of K_i for phosphoglycolate found for the mutant and wild-type enzymes. Under our assay conditions, these values are $1.8 \pm 0.2 \times 10^{-5}$ M and $1.9 \pm 0.2 \times 10^{-5}$ M, respectively. Previously reported K_i values for this substance range from 4×10^{-7} to 6×10^{-6} M; however, the K_i is known to be very sensitive to small changes in pH and ionic strength (36). Our result suggests

Table 1. Kinetic constants for wild-type and mutant TIM

Constant	Wild type	Mutant	Wild type/ mutant
$\vec{k}_{cat}$	4.3×10^2 sec^{-1}	1.8 ± 0.1 sec^{-1}	240
$\vec{K}_m$	9.7×10^{-4} M	$1.8 \pm 0.2 \times 10^{-3}$ M	0.54
$\vec{K}_{m(unhyd)}$	5.7×10^{-4} M	$1.1 \pm 0.1 \times 10^{-3}$ M	0.54
$\overleftarrow{k}_{cat}$	4.3×10^3 sec^{-1}	2.8 ± 0.2 sec^{-1}	1500
$\overleftarrow{K}_m$	4.7×10^{-4} M	$1.3 \pm 0.1 \times 10^{-4}$ M	3.6
$\overleftarrow{K}_{m(unhyd)}$	1.8×10^{-5} M	$5.1 \pm 0.3 \times 10^{-6}$ M	3.6
K_{eq}	20.8	21 ± 3	1.0
$\overleftarrow{R}$	2.2×10^{-2}*	1.1×10^{-5}*	2000
$\vec{R}$	7.1×10^{-1}	2.4×10^{-3}*	300

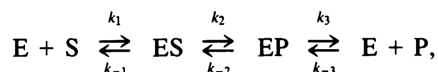
Values for wild-type TIM are from ref. 10; mutant TIM values were determined with the aid of program HYPERO (32). $\vec{K}_{m(unhyd)} = \vec{K}_m/(1 + 1.44^{-1})$; $\overleftarrow{K}_{m(unhyd)} = \overleftarrow{K}_m/(1 + 0.039^{-1})$ (18); $K_{eq} = (\overleftarrow{k}_{cat}/\overleftarrow{K}_m)(\vec{K}_m/\vec{k}_{cat})$. See *Discussion* for definition of $\vec{R}$ and $\overleftarrow{R}$.
*Assuming k_1(mutant) = k_{-3}(mutant) = k_1(wild type) = 3.7×10^8 M^{-1}·sec^{-1}.

that glutamic acid-165 plays at best a minor role in binding the enediol(ate) intermediate.

DISCUSSION

By effecting a subtle alteration in a residue crucial to the catalytic function of TIM, we hoped to perturb the enzyme in such a way that it still retained some catalytic activity. On the basis of earlier chemical modifications of the protein (4–6, 37, 38) and analysis of the crystal structure of the chicken and yeast proteins, we predicted that the glutamic acid-165 → aspartic acid mutant would be much less efficient than the wild-type enzyme. This expectation has been realized, and the enzyme with this change, although severely crippled by a shift of the critical carboxyl group, is still amenable to kinetic analysis.

Any single substrate enzymatic reaction can be depicted as a three step process



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P,$$

where the second step subsumes all of the catalytic events. The steady-state kinetic data that we have obtained allow us to define all of the rate constants in this simplified scheme, if we assume values for the "on" rates (k_1 and k_{-3}) for the two substrates. On the basis of the earlier studies on this enzyme (10) the "on" rates can reasonably be taken to be diffusive, at $\approx 3.7 \times 10^8$ $M^{-1} \cdot sec^{-1}$. The rate constants, k_{-1}, k_2, k_{-2}, and k_3 can be expressed in terms of k_1, k_{-3}, and the four steady-state parameters. Thus, if we assume values for k_1 and k_{-3} we can calculate the other four rate constants from the following equations:

$$k_2 = \frac{\overrightarrow{k}_{cat}}{1 - \overrightarrow{R}} \qquad k_{-2} = \frac{\overleftarrow{k}_{cat}}{1 - \overleftarrow{R}}$$

$$k_{-1} = \left[\frac{1}{\overrightarrow{R}} - 1\right]\left[k_2 + k_{-2}\right]$$

$$k_3 = \left[\frac{1}{\overleftarrow{R}} - 1\right]\left[k_2 + k_{-2}\right]$$

where

$$\overrightarrow{R} = \frac{\overrightarrow{k}_{cat} + \overleftarrow{k}_{cat}}{k_1 \overrightarrow{K}_m} \text{ and } \overleftarrow{R} = \frac{\overrightarrow{k}_{cat} + \overleftarrow{k}_{cat}}{k_{-3} \overleftarrow{K}_m}.$$

The sizes of the ratios $\overrightarrow{R}$ and $\overleftarrow{R}$ indicate whether the enzyme is of the classical Michaelis–Menten type, where the formation of the central complexes is fast relative to the rate of interconversion of these complexes. When both $\overrightarrow{R}$ and $\overleftarrow{R}$ are much smaller than 1, k_2 is equal to $\overrightarrow{k}_{cat}$, k_{-2} equals $\overleftarrow{k}_{cat}$, $\overrightarrow{K}_m$ equals $\overrightarrow{K}_s$, and $\overleftarrow{K}_m$ equals $\overleftarrow{K}_s$, where $\overrightarrow{K}_s = (k_{-1}/k_1)$ and $\overleftarrow{K}_s = (k_3/k_{-3})$. $\overrightarrow{R}$ and $\overleftarrow{R}$ for the mutant enzyme are both much less than 1, while for wild-type TIM these ratios are appreciable (Table 1). Using the values listed in Table 1 and the equations shown above, we calculated the rate constants for the three-step path (Table 2). Fig. 4 plots the simplified free-energy profiles, based on these rate constants, for the mutant TIM (dotted line) and for the wild-type enzyme (solid line). By changing glutamic acid-165 to aspartic acid, the level of the transition state for substrate interconversion has been affected almost exclusively: the free energies of bound substrate and of bound product are minimally perturbed. In the complete free energy profile [that is, where the bound enediol(ate) is included as a reaction intermediate], substrate interconversion is separated into two steps, representing the

Table 2. Calculated rate constants*

Rate constant	Wild type	Mutant
k_1	3.7×10^8 $M^{-1} \cdot sec^{-1}$†	3.7×10^8 $M^{-1} \cdot sec^{-1}$†
k_{-1}	2.6×10^5 sec^{-1}	3.3×10^5 sec^{-1}
k_2	1.5×10^3 sec^{-1}	1.8 sec^{-1}
k_{-2}	4.4×10^3 sec^{-1}	2.8 sec^{-1}
k_3	2.4×10^3 sec^{-1}	1.9×10^3 sec^{-1}
k_{-3}	3.7×10^8 $M^{-1} \cdot sec^{-1}$	3.7×10^8 $M^{-1} \cdot sec^{-1}$†

*For the simplified reaction $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P$.

†Assumed to equal the observed value for k_{-3} (wild type).

deprotonation of substrate and the protonation of the enediol(ate). The fact that both the mutant and the wild-type enzymes have the same K_i for phosphoglycolate, which is a likely analogue of the enediol(ate) intermediate, suggests that glutamic acid-165 plays only a minor role in binding this intermediate.

The wild-type enzyme has been called a perfect catalyst (2), because the rate-limiting step of the overall reaction is a diffusive step involving the encounter of GAP, the thermodynamically less stable substrate, and the enzyme. In contrast, the mutant enzyme conforms to the classical Michaelis–Menten mechanism in which the catalytic steps are much slower than the binding steps. Our data add to the evidence that glutamic acid-165 is the residue that shuttles protons between C-1 and C-2 of the triosephosphates. We believe that the altered kinetic properties of the mutant enzyme are due almost entirely to increases in the transition state free energies for the protonation and deprotonation steps and that the free energies of the bound intermediates are affected only minimally.

Our work on site-directed mutagenesis of TIM adds to the rapidly growing evidence that this technique, when combined with classical enzymological and structural analysis, can be a powerful tool for dissecting the molecular basis of enzymatic catalysis. The advantages of using TIM for such studies lie in the depth of our existing kinetic and structural knowledge of the enzyme, the possibility of combining high resolution structural analysis with detailed examination of the enzyme's dynamic properties, and the ability to screen for activity by a genetic complementation test.

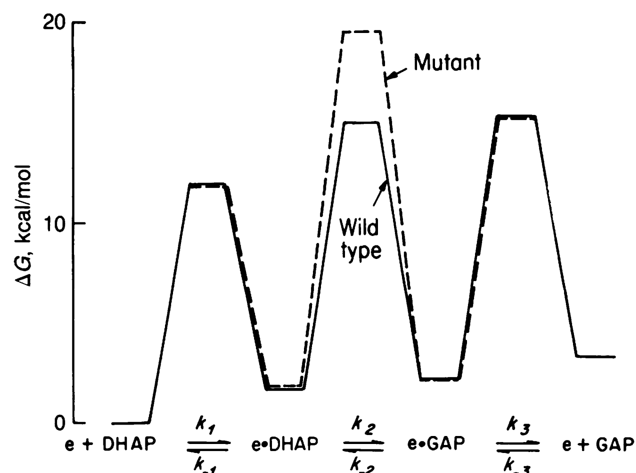


FIG. 4. Gibbs free-energy profiles for the simplified (three step) reaction path for mutant (dashed line) and wild-type TIM (solid line). The ΔG values were determined from rate constants in Table 2. A standard state of 40 μM has been assumed (39).

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