How Can a Catalytic Lesion Be Offset? The Energetics of Two Pseudorevertant Triosephosphate Isomerases†

Stephen C. Blacklow and Jeremy R. Knowles*
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
Received October 24, 1989; Revised Manuscript Received January 4, 1990

ABSTRACT: The reaction energetics of four triosephosphate isomerase mutants are compared with those of the wild-type enzyme. The two primary mutants, E165D and H95N, contain site-specific alterations of active site residues. In one case the active site base has been altered (E165D), and in the other, an active site electrophile has been removed (H95N), yet the major effect in each case is the relative destabilization of the transition states for the two chemical (enolization) steps that constitute the catalytic reaction. When the genes encoding each of these sluggish mutant isomerases were subjected to random mutagenesis using chemical reagents and a selection for isomerases of increased catalytic potency was performed, pseudorevertant enzymes with dramatic increases in activity were found. Remarkably, the same second-site suppressor locus partially corrects each lesion. The E165D,S96P pseudorevertant is a 20-fold better catalyst than the E165D mutant from which it is derived, and the H95N,S96P pseudorevertant is about 60 times more active than its H95N parent. The S96P substitution thus increases the catalytic activity in each of two different contexts, H95N and E165D. The energetic consequences of the S96P change are surprisingly similar in each pseudorevertant. The H95N,S96P enzyme is more effective than H95N at stabilizing the intermediate enediol(ate) phosphate and its flanking transition states. The E165D,S96P enzyme likewise stabilizes the transition states for enolization better than E165D, and this pseudorevertant also forms a tighter enzyme–dihydroxyacetone phosphate complex than its parent. These data show how, in these two cases, the catalytic potency of sluggish mutant enzymes can be improved by second-site changes. The results thus provide the beginnings of a detailed understanding of the kinetic refinement of enzyme catalysts.

The techniques of site-directed and random mutagenesis have become enticing methods for exploring the structure–function relationship in proteins (Leatherbarrow & Fersht, 1986; Shaw, 1987; Knowles, 1987; Gerlt, 1987). In particular, the behavior of site-directed mutants chosen on the basis of structural and mechanistic understanding of the target enzyme can provide useful information about the relevance of particular amino acids or of regions of the enzyme to stability or catalysis. It has become clear, however, that the alteration of residues directly involved in catalysis may have subtle mechanistic consequences (Howell et al., 1987; Nickbarg et al., 1988), and it is therefore imperative that mutant enzymes be fully characterized in structural and catalytic terms.

The enzyme triosephosphate isomerase (TIM) is particularly appropriate for analysis by mutagenic techniques because the wild-type enzyme is already so well studied. The enzyme from chicken muscle is a dimer of subunit molecular weight 26 500 (Putman et al., 1972), and the catalyzed isomerization, outlined in Figure 1, involves the abstraction of the *pro-R* proton of dihydroxyacetone phosphate to form an enediol (or enediolate) phosphate intermediate. This intermediate then collapses with reprotonation at C-2 to give the product D-glyceraldehyde 3-phosphate (Rieder & Rose, 1956, 1959; Bloom & Topper, 1956). The structures of several enzyme crystals have been solved to high resolution: for the chicken enzyme to 2.5 Å (Banner et al., 1975), for the unliganded yeast enzyme to 1.9 Å, and for the yeast enzyme containing bound phosphoglycolohydroxamate to 1.9 Å (Davenport, 1986; Alber et al., 1987). A schematic picture of the active site region is shown in Figure 2. There is substantial evidence that the base required for proton abstraction is a glutamate residue at position 165 (Hartman, 1968; Waley et al., 1970; de la Mare et al., 1972), and electrophilic catalysis is thought to be provided by histidine-95 and/or lysine-13. Early efforts to define the individual contributions of active site residues focused on the contributions of E165 and H95. Glutamate-165 was changed to aspartate, and the structure and catalytic behavior of this E165D mutant was studied (Straus et al., 1985; Raines et al., 1986). From the difference electron density map between the wild-type enzyme and the E165D mutant, it appears that the carboxylate group moves by about 1 Å in E165D and that there is very little residue movement elsewhere in the protein (E. Lolis and G. Petsko, personal communication). Kinetic characterization of the mutant E165D enzyme showed the importance of the positioning of the base at the active site. While the rate of reaction catalyzed by the wild-type enzyme is encounter controlled (Albery & Knowles, 1976b; Blacklow et al., 1988), the rate-limiting step for the E165D mutant is the enolization of dihydroxyacetone phosphate (see Figure 1), and the overall reaction rate (that is, the value of k_{cat}/K_m) is reduced by more than 300-fold (Raines et al., 1986). In other experiments, the contribution of H95 to the catalyzed reaction was assessed by generating the mutant H95Q (Nickbarg et al., 1988). This mutation slows the rate of the catalyzed reaction by 400-fold, and the mechanistic pathway followed by the mutant enzyme is subtly altered. To clarify further the role of H95 in the catalytic mechanism, we have now analyzed the behavior of a mutant enzyme in which this histidine has been changed to asparagine (H95N).

In an effort to assess how readily these catalytic lesions can be reversed, we searched for second-site suppressors (pseudorevertants) from the two sluggish isomerase mutants, E165D and H95N, that have increased catalytic effectiveness. The cloned genes of these two mutant isomerases were therefore subjected to random mutagenesis using a range of chemical

† This work was supported by the National Science Foundation, the National Institutes of Health, and Merck Sharp & Dohme.

0006-2960/90/0429-4099$02.50/0

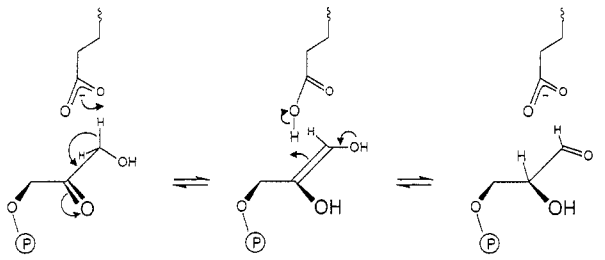

FIGURE 1: Interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, catalyzed by triosephosphate isomerase.

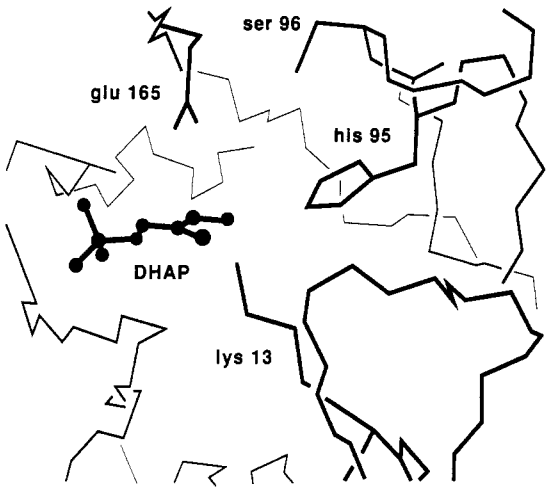

FIGURE 2: Active site of wild-type triosephosphate isomerase. The coordinates are those of the native chicken muscle enzyme (Banner et al., 1976). Dihydroxyacetone phosphate has been positioned in the active site to minimize nonbonding contacts between the enzyme and the substrate.

reagents (Myers et al., 1985), and a library of transformants carrying changes at second sites was produced (Hermes et al., 1987a). From this library, those colonies that produced isomerases of increased specific catalytic activity were selected and purified. Surprisingly, *in each case*, the same second-site suppressor mutation, S96P, was found. That is, the catalytic activity of the E165D isomerase (which has a k_{cat}/K_m value that is about 300 times smaller than that of the wild-type enzyme) is increased by nearly 20-fold in the double mutant E165D,S96P. Analogously, the activity of the H95N isomerase (which has a value of k_{cat}/K_m that is about 3500 times smaller than that of the wild type) rises by about 60-fold in the pseudorevertant H95N,S96P. Now, from all that is known about the structure and catalytic behavior of triosephosphate isomerase, E165 is the base that abstracts the carbon-bound protons from each of the substrates, and H95 fulfills a role as a catalytic electrophile. So it is at first sight extraordinary that two such different lesions at the active site should each be partially suppressed by the same second-site mutation. Could the change of serine-96 to proline effect some sort of "global" suppression (Shortle & Lin, 1985) that improves the catalytic prowess of *all* triosephosphate isomerases? To answer this question, we introduced the S96P change directly into the wild-type enzyme. This mutant enzyme was found to be considerably less active than the wild type, showing the *non*-additivity of amino acid substitutions at these three sites in the protein. To explore these activity relationships further and to define the kinetic consequences of the structural alterations, five enzymes (the two primary mutants E165D and H95N, both of the pseudorevertants, and the S96P mutant) have been purified and kinetically characterized. The two pseudorevertants have been analyzed by the isotopic substitution methods described earlier (Albery & Knowles, 1976a) to determine their free-energy profiles. These profiles are compared both to that of the wild-type enzyme and to those of the sluggish mutants from which they are derived, to provide a better understanding of how they differ as catalysts from the wild type and from their mutant parents.

Experimental Procedures

Materials. Escherichia coli strain DF502, a streptomycin-resistant strain of *E. coli* that lacks the endogenous *E. coli* triosephosphate isomerase [constructed in a manner analogous to that of DF500 by D. Fraenkel (Babul, 1978)], was a generous gift from D. Fraenkel. The plasmids bearing mutant triosephosphate isomerases of the chicken gene were constructed from plasmid pX1 (Straus & Gilbert, 1985).

Native chicken triosephosphate isomerase was purified by J. G. Belasco according to Putman et al. (1972). Aldolase (rabbit muscle), enolase (rabbit muscle), pyruvate kinase (rabbit muscle), phosphoglycerate kinase (yeast), triosephosphate isomerase (bakers' yeast), and phosphoglycerate mutase (rabbit muscle) were obtained as ammonium sulfate suspensions from Boehringer-Mannheim (Indianapolis, IN). α-Glycerophosphate dehydrogenase (rabbit muscle) and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were also obtained from Boehringer-Mannheim. Traces of contaminating triosephosphate isomerase activity were removed from the dehydrogenases by treatment with bromohydroxyacetone phosphate as described by de la Mare et al. (1972). Excess bromohydroxyacetone phosphate was then removed by ultrafiltration through Amicon Centricon 10 ultrafiltration cones (at 5000*g*, repeated twice) after dilution with 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA[1] (10 mM) and β-mercaptoethanol (1 mM), at 4 °C.

(*R,S*)-Glyceraldehyde 3-phosphate (diethyl acetal, monobarium salt), 2,3-diphospho-(*R*)-glycerate [pentakis(cyclohexylammonium) salt], NAD^+, NADH (disodium salt), ATP (disodium salt, grade 1), (*R,S*)-glycerol phosphate (disodium salt, hexahydrate), 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). The diethyl acetal of (*R,S*)-glyceraldehyde phosphate was hydrolyzed before use according to the manufacturer's instructions. DEAE-cellulose (DE-52) was from Whatman Inc. (Clifton, NJ). AG1 (Cl^- form, 200–400 mesh, 8% cross-linked) was from Bio-Rad (Richmond, CA). Dihydroxyacetone phosphate was prepared by R. Raines from 3-chloro-1,2-propanediol according to the method of Ballou (1960) or was purchased from Sigma. 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. Raines using the method of Herlihy et al. (1976). Tritiated water (4.5 Ci/mL) was from Amersham (Chicago, IL). Phospho[1-^{14}C]glycerate (14.1 Ci/mol), prepared according to the method of Guilford et al. (1987), was a gift from D. L. Pompliano. [1-^{14}C]Glyceraldehyde 3-phosphate and [1-^{14}C]dihydroxyacetone phosphate were prepared enzymatically from phospho[1-^{14}C]glycerate as described by Nickbarg et al. (1988). Flo-Scint III scintillation cocktail was from Radiomatic Instruments and

[1] Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; DEAE, diethylaminoethyl; DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetate; GAP, glyceraldehyde phosphate; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; QAE, [diethyl(2-hydroxypropyl)amino]ethyl; Tris, tris(hydroxymethyl)aminomethane.

Chemical Co. (Tampa, FL); Scintiverse-II scintillation cocktail was from Fisher (Medford, MA). Bactotryptone and Bacto yeast extract were from Difco Labs (Detroit, MI). Ammonium sulfate was special enzyme grade from Schwarz/Mann (Cambridge, MA). All other chemicals and reagents were of the best available commercial grades.

Methods. Mutant isomerases were purified as described (Raines et al., 1986). The genes encoding each of the triosephosphate isomerase mutants (E165D and H95N) were subjected to random mutagenesis with chemical reagents using the method of Myers et al. (1985). Selection for isomerases of increased catalytic activity was performed on M63 minimal plates (Miller, 1972) with glycerol as the sole carbon source, as described (Hermes et al., 1987a).

Samples (5–1000 μL) for radiochemical analysis were dissolved in scintillation cocktail (5 mL) and counted in a Beckman LS1801 automatic liquid scintillation counter. Scintillation counting for both ^3H and ^{14}C was done after calibration with Beckman counting standards, the double-label counting programs supplied with the instrument being used. 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 absorbance were measured on a Hewlett-Packard 8452A diode array spectrophotometer thermostated with a Brinkmann RMS 6 temperature controller. The concentration of a solution of phosphoglycolohydroxamate was determined by assay of the inorganic phosphate released when the sample was treated with alkaline phosphatase. Inorganic phosphate was determined by the method of Ames (1966). Methylglyoxal was quantitated by end-point assay using glyoxalase I as described by Racker (1957).

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 16 min) of triethylammonium formate buffer, pH 3.8, at a flow rate of 2 mL/min. The 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.

Steady-State Kinetics. Enzyme assays, based on the method of Putman et al. (1972), were performed at 30 °C in 0.1 M triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM). The validity of the assays was checked by ensuring the linear dependence of the observed initial rate on the concentration of triosephosphate isomerase at both high and low substrate concentrations. An extinction coefficient for NADH of 6220 M^{-1} cm^{-1} at 340 nm was assumed (Horecker & Kornberg, 1948). The values of k_{cat} and of K_m for each substrate were determined with the computer program HYPERO (Cleland, 1979).

Stoichiometry of Product Formation. The amount of glyceraldehyde 3-phosphate consumed under irreversible conditions by the mutant enzymes (E165D,S96P, H95N, H95N,S96P, and S96P) was compared to the amount of dihydroxyacetone phosphate produced. The reaction mixture contained (in 1.05 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.6, EDTA (10 mM), isomerase-free α-glycerophosphate dehydrogenase (0.1 mg), NADH (0.12 mg), and glyceraldehyde 3-phosphate (0.15 μmol). The absorbance at 340 nm was recorded. The reaction was initiated by the addition of a mutant isomerase (~2 units). The consumption of glyceraldehyde 3-phosphate was allowed to continue until there was no further change in A_{340nm}. The change in absorbance so determined was compared to that found when the wild-type isomerase was used. To determine the amount of methylglyoxal produced by the H95N mutant isomerase, the reaction mixture was passed through an Amicon Centricon-10 ultrafiltration cone at 5000*g* to remove isomerase and α-glycerophosphate dehydrogenase. The amount of methylglyoxal appearing in the filtrate was assayed with glyoxalase I and reduced glutathione (Racker, 1957).

Fate of the Tritium Label from Substrate [1(R)-^3H,1-^{14}C]Dihydroxyacetone Phosphate. The reaction mixture contained (in 1.65 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.6, EDTA (10 mM), isomerase-free glyceraldehyde-3-phosphate dehydrogenase (80 units), NAD^+ (5 mg), sodium arsenate (10 mM), and [1(*R*)-^3H,1-^{14}C]dihydroxyacetone phosphate (1 μmol, 0.036 μCi of ^{14}C, $^3H/^{14}C$ ~ 5.0). The solution was mixed and equilibrated at 30 °C. A reference sample (200 μL) was removed and quenched in 1 N HCl (25 μL) before storage at −70 °C for later analysis. The A_{340nm} was determined. The reaction was then initiated by the addition of mutant isomerase (2.5 units). A portion of the reaction mixture was transferred to a short-path (2 mm) optical cuvette for monitoring at 340 nm to determine the extent of reaction. At four intervals, samples (200 μL) were withdrawn and quenched into 1 N HCl (25 μL). At the completion of the reaction, the sample in the optical cuvette was quenched and stored. After quenching, solvent was removed from each sample by bulb-to-bulb distillation. The sample residues were taken up in water (0.3 mL), filtered through Amicon Centricon-10 ultrafiltration cones at 5000*g* to remove isomerase and glyceraldehyde-3-phosphate dehydrogenase, and adjusted to pH 4 with 0.1 N NaOH. The mixtures of radiolabeled 3-phosphoglycerate and dihydroxyacetone phosphate were then separated by HPLC and analyzed for ^3H and ^{14}C as described under Methods.

Appearance of Solvent Tritium in Remaining Substrate [1-^{14}C]Dihydroxyacetone 3-Phosphate and in Product. Each mutant isomerase catalyzed reaction was run under conditions similar to those used by Maister et al. (1976). The reaction mixture contained (in 1.50 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.6, EDTA (10 mM), NAD^+ (3 mM), sodium arsenate (10 mM), glyceraldehyde-3-phosphate dehydrogenase (80 units), tritiated water (45 mCi), and [1-^{14}C]dihydroxyacetone phosphate (1 mM, 0.036 Ci/mol). The initial absorbance at 340 nm was determined, and duplicate samples (10 μL) were removed and diluted into water (1.0 mL) for later determination of the solvent tritium specific radioactivity. A third sample (200 μL) was removed, brought to pH 2 with 1 N HCl (25 μL), quickly frozen in liquid nitrogen, and stored at −70 °C. The reaction mixture was equilibrated at 30 °C, and the reaction was initiated by the addition of mutant isomerase (2.5 units). A portion (500 μL) of the reaction mixture was transferred to a short-path (2 mm) optical cuvette in order to monitor the extent of reaction at 340 nm. The remainder of the reaction mixture was separately incubated at 30 °C. At four stages in the reaction, samples (200 μL) were removed from the reaction mixture and quenched into 1 N HCl (25 μL). Each sample was frozen in liquid nitrogen and stored at −70 °C. Solvent was then removed from each sample by bulb-to-bulb distillation. The residues were dissolved in 0.8 mM HCl (0.5 mL), and the

Table I: Steady-State Kinetic Parameters for Wild-Type and Mutant Triosephosphate Isomerases

parameter[a]	enzyme					
	wild type	H95N	H95N,S96P	E165D	E165D,S96P	S96P
k_{cat}^S (s^{-1})	600	0.16	6.8	4.1	3.4	11
K_m^S (mM)	0.65	0.59	0.40	1.2	0.053	0.24
K_m^S(unhydrated) (mM)	0.38	0.35	0.24	0.71	0.031	0.14
$(k_{cat}^S/K_m^S)_{rel}$	1.0	2.9×10^{-4}	1.8×10^{-2}	3.7×10^{-3}	6.9×10^{-2}	5.0×10^{-2}
k_{cat}^P (s^{-1})	8300	0.46	29	4.2	68	64
K_m^P (mM)	0.42	0.081	0.083	0.078	0.066	0.087
K_m^P(unhydrated) (mM)	0.016	0.0030	0.0031	0.0029	0.0025	0.0033
$(k_{cat}^P/K_m^P)_{rel}$	1.0	2.9×10^{-4}	1.8×10^{-2}	2.7×10^{-3}	5.2×10^{-2}	4.3×10^{-2}
$K_i(HAsO_4^{2-})$ (mM)	11	3.0	0.074	12	2.1	0.7
K_i(phosphoglycolohydroxamate) (μM)	7.1	950	110	22	9.8	0.5

[a] Values for the unhydrated forms of the substrates are calculated as described in Albery and Knowles (1976b). k_{cat}/K_m values are quoted relative to the wild-type enzyme. k_{cat}^S and K_m^S values refer to dihydroxyacetone phosphate as substrate; k_{cat}^P and K_m^P relate to D-glyceraldehyde 3-phosphate as substrate.

solvent was again removed by bulb-to-bulb distillation. The residues were each dissolved in water (0.5 mL), and the solvent was again removed. The samples were then dissolved in water (0.5 mL), and coupling enzyme and isomerase were removed from these solutions by ultrafiltration. The samples were adjusted to pH 4 with 0.1 N NaOH and diluted to a total volume of 1.0 mL with water. 3-Phosphoglycerate and dihydroxyacetone phosphate were then separated by HPLC and the $^3H/^{14}C$ ratio was determined as described under Methods.

Appearance of Solvent Tritium in Remaining Substrate [1-^{14}C]Glyceraldehyde Phosphate and in Product. Each mutant isomerase-catalyzed reaction was run under conditions similar to those used by Fletcher et al. (1976). The reaction mixture contained (in 1.60 mL) 100 mM triethanolamine hydrochloride buffer, pH 7.6, EDTA (10 mM), NADH (2.2 mg), α-glycerophosphate dehydrogenase (200 units), (*R*,*S*)-[1-^{14}C]glyceraldehyde 3-phosphate (2 mM, 0.04 Ci/mol), and tritiated water (27 mCi). After equilibration to 30 °C, two samples (10 μL) were removed and diluted into water (1.0 mL) for later assay to determine the solvent tritium specific radioactivity. A larger sample (200 μL) was also removed and quenched into 1 N HCl (25 μL) and immediately frozen in liquid nitrogen and stored. The isomerase reaction was then initiated in the reaction mixture by the addition of mutant isomerase (0.37 unit). The extent of reaction was monitored at 340 nm by use of a portion of the reaction mixture (500 μL) in a short-path (2 mm) optical cuvette. At four stages in the reaction, samples (200 μL) were removed and quenched into 1 N HCl (25 μL). The quenched samples were immediately frozen in liquid nitrogen and stored. The solution in the optical cuvette was also quenched with 1 N HCl (75 μL) and stored. Solvent was then removed from each sample by bulb-to-bulb distillation. The sample residues were dissolved in 0.8 mM HCl (0.5 mL), and the solvent was again removed by distillation. This was repeated once more. The sample residues were then redissolved in 0.8 mM HCl (0.5 mL) and the enzymes removed by ultrafiltration. The remaining glyceraldehyde phosphate in the samples was then converted into 3-phosphoglycerate. Each sample was neutralized with NaOH (0.1 N); the solution was transferred to an optical cuvette and then diluted with water to a volume of 1.0 mL. NAD^+ (0.1 mg) and sodium arsenate (5 μmol) were added, and the initial absorbance at 340 nm was determined. Isomerase-free glyceraldehyde-3-phosphate dehydrogenase (8 units) was then added, and the reaction was followed to completion by monitoring the absorbance at 340 nm. The glyceraldehyde-3-phosphate dehydrogenase was removed from each sample by ultrafiltration, and the 3-phosphoglycerate and glycerol phosphate were then separated by HPLC and their specific radioactivity was determined as described under Methods.

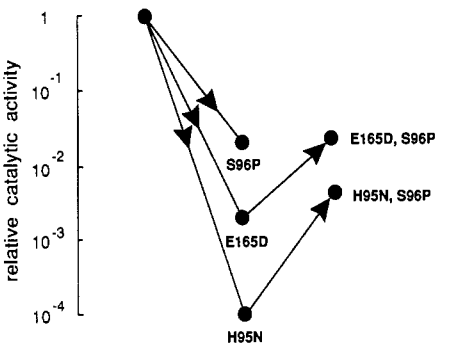

FIGURE 3: Catalytic activity, relative to the wild-type enzyme and expressed as k_{cat}/K_m, of the H95N,S96P and E165D,S96P pseudorevertants obtained by chemical mutagenesis of the genes encoding two sluggish triosephosphate isomerase mutants: H95N and E165D. For comparison, the relative catalytic activities of the H95N, S96P, and E165D mutant enzymes are also shown.

Results

Steady-State Kinetics. Each mutant enzyme was purified to homogeneity as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970). The subunit M_r of each mutant was indistinguishable from that of the wild-type chicken enzyme. The steady-state kinetic parameters for these enzymes are listed in Table I.[2] Also included in this table are the inhibition constants for inorganic arsenate, a necessary component of the assay in the determination of K_m for DHAP, and for phosphoglycolohydroxamate, which is an analogue of the enediol(ate) phosphate intermediate. The relative values of k_{cat}/K_m are shown in Figure 3.

Stoichiometry of Product Formation. The amount of product formed by the mutant enzymes was assessed when either DHAP or (*R*,*S*)-GAP was used as the substrate. The amount of substrate DHAP converted to GAP (and, by the coupling enzyme in situ, to 3-phosphoglycerate) by each of the mutant enzymes was close to stoichiometric. When GAP was the substrate, the amount of product DHAP (which was converted by the coupling enzyme in situ to glycerol phosphate) formed from the H95N,S96P and E165D,S96P enzymes was close to stoichiometric, but the H95N enzyme only produced 67% of the stoichiometric quantity of glycerol phosphate. When an isolated product mixture was incubated in the presence of reduced glutathione and the enzyme glyoxalase

[2] The rate of formation of triose phosphate product was used to determine the values of the kinetic parameters reported in Table I. This distinction is important, since the rate of formation of DHAP by the H95N enzyme is only two-thirds of the rate of consumption of GAP (see Stoichiometry of Product Formation).

Table II: Variation of the Tritium Content of Substrate [1(*R*)-^{3}H,1-^{14}C]Dihydroxyacetone Phosphate (s/s_0) or of Product [2-^{3}H,1-^{14}C]Glyceraldehyde Phosphate (p/s_0) in the Reactions Catalyzed by the Mutant Isomerases H95N, H95N,S96P, and E165D,S96P for Different Extents of Reaction ($1 - r$)

H95N enzyme			H95N,S96P enzyme			E165D,S96P enzyme		
$1 - r$	s/s_0	p/s_0	$1 - r$	s/s_0	p/s_0	$1 - r$	s/s_0	p/s_0
0	1.0	0	0	1.0	0	0	1.0	0
0.49	1.80	0	0.49	1.81	0	0.36	1.45	0.0086
0.69	2.82	0	0.73	3.09	ND	0.52	1.86	0.0087
0.96	ND[a]	0	0.75	3.21	ND	0.62	2.23	0.0076
			0.86	5.17	0.001	0.73	3.07	0.010
			0.88	5.59	0	0.9	7.3	0.013
						0.9	6.9	0.014

[a] ND, not determined.

Table III: Enzyme-Catalyzed Incorporation of Solvent Tritium into Remaining Substrate [1(*R*)-^{3}H,1-^{14}C]Dihydroxyacetone Phosphate (s/x) or into Product [2-^{3}H,1-^{14}C]Glyceraldehyde Phosphate (p/x) for Different Extents of Reaction ($1 - r$) for the Reactions Catalyzed by the Mutant Isomerases H95N, H95N,S96P, and E165D,S96P

H95N enzyme			H95N,S96P enzyme			E165D,S96P enzyme		
$1 - r$	s/x	p/x	$1 - r$	s/x	p/x	$1 - r$	s/x	p/x
0	0	0	0	0	0	0	0	0
0.15	0.00	0.15	0.10	0	0.59	0.26	0.037	0.92
0.34	0.004	0.13	0.22	ND	0.58	0.34	ND	0.97
0.45	ND[a]	0.15	0.37	0.014	0.64	0.58	0.06	0.97
0.55	0.003	0.155	0.35	0.024	0.65	0.68	0.11	0.97
0.66	0.004	ND	0.57	0.054	0.68	0.79	0.18	1.01
0.83	0.017	0.14	0.79	0.166	0.73	0.81	ND	0.91
0.90	0.038	0.14	0.85	ND	0.71			
0.91	0.07	0.14						

[a] ND, not determined.

I, the amount of methylglyoxal present was found to account entirely for the difference between the GAP consumed and the DHAP formed. When [1-^{14}C]GAP was the substrate for the H95N enzyme, the fraction of ^{14}C counts appearing as methylglyoxal in the chromatographed product was again found to be about one-third of the product mixture, the other two-thirds appearing as product glycerol phosphate.

Isotopic Substitution Experiments. The extent of tritium transfer from doubly labeled [1(*R*)-^{3}H,^{14}C]dihydroxyacetone phosphate to product glyceraldehyde phosphate, and thus ultimately to 3-phosphoglycerate (expressed as the ratio of the specific radioactivity of the ultimate product p to the specific radioactivity of the substrate at the start of the reaction s_0), was investigated for the mutants H95N, H95N,S96P, and E165D,S96P. The enrichment of tritium in the remaining substrate (as measured by s/s_0) was also determined in this experiment. The ^{14}C label was included to facilitate determination of the extent of reaction (from the distribution of ^{14}C radioactivity), and the relative ^{3}H specific radioactivities (i.e., s/s_0 and p/s_0) were easily determined. The values of s/s_0 and of p/s_0 determined at several extents of reaction ($1 - r$) for the H95N, H95N,S96P, and E165D,S96P mutants are reported in Table II, and the former are plotted for comparison with the wild-type data in Figure 4.

The appearance of solvent tritium in remaining substrate [1-^{14}C]dihydroxyacetone phosphate and in the product was determined at several extents of reaction for the mutant isomerases H95N, H95N,S96P, and E165D,S96P. Values of the specific radioactivity of substrate and of product were determined from the ^{3}H/^{14}C ratios. For each mutant, the values of the specific radioactivity of substrate (s) divided by the specific radioactivity of the solvent (x) are presented at several extents of reaction ($1 - r$) in Table III; these results are compared with the data from the wild-type enzyme in Figure 5. For each mutant, the specific radioactivity of product (p) divided by the specific radioactivity of the solvent (x) is listed for several extents of reaction in Table III.

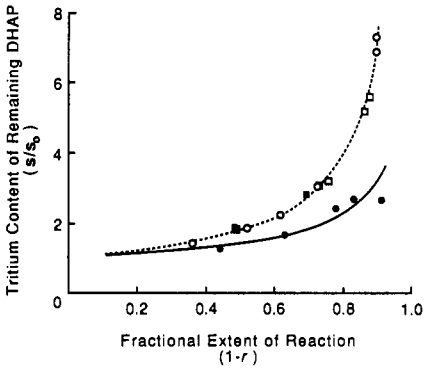

FIGURE 4: Variation in specific radioactivity of the substrate [1-(*R*)-^{3}H]dihydroxyacetone phosphate (s) with the fractional extent of reaction ($1 - r$) for the wild-type (●) and for the H95N (■), H95N,S96P (□), and E165D,S96P (○) triosephosphate isomerases. Data for the wild-type enzyme are from Herlihy et al. (1976). The fitted line (solid) for the wild-type enzyme data is derived from the equation $s/s_0 = r^{A_6'-1}$ (Albery & Knowles, 1976a), with $A_6' = 0.50$ (Herlihy et al., 1976). The fitted line (dotted) for the data from all of the mutant enzymes is from the same equation, with $A_6' = 0.15$.

With [1-^{14}C]glyceraldehyde phosphate as substrate, several exchange-conversion experiments were performed with the three mutant isomerases H95N, H95N,S96P, and E165D,-S96P. Data points for at least four extents of reaction were obtained for each enzyme, in addition to a reference value determined before initiation of the reaction by the mutant isomerase. The amount of solvent tritium appearing in remaining substrate [1-^{14}C]GAP (which was converted to 3-phosphoglycerate before analysis) was determined for each mutant enzyme at several extents of reaction from the ^{3}H/^{14}C ratio of the isolated 3-phosphoglycerate. For each of these mutant enzymes, the values for the specific radioactivity of substrate (p) relative to the specific radioactivity of solvent (x) are reported in Table IV. These results are contrasted with the data from the wild-type enzyme in Figure 6. For each enzyme, the amount of solvent tritium appearing in

Table IV: Enzyme-Catalyzed Incorporation of Solvent Tritium into Remaining Substrate [2-^{3}H,1-^{14}C]Glyceraldehyde Phosphate (p/x) or into Product [1(R)-^{3}H,1-^{14}C]Dihydroxyacetone (s/x) for Different Extents of Reaction ($1 - r$) for the Reactions Catalyzed by the Mutant Isomerases H95N, H95N,S96P, and E165D,S96P

H95N enzyme			H95N,S96P enzyme			E165D,S96P enzyme		
$1 - r$	p/x	s/x	$1 - r$	p/x	s/x	$1 - r$	p/x	s/x
0	0	0	0	0	0	0	0	0
0.12	0.33	ND[a]	0.14	0.22	ND	0.11	0.16	0.064
0.20	0.53	0.092	0.30	0.66	ND	0.12	0.17	0.057
0.39	1.04	0.075	0.53	0.96	0.10	0.31	0.44	0.070
0.41	1.01	0.092	0.77	ND	0.10	0.48	0.60	ND
0.61	1.44	0.084	0.92	ND	0.093	0.60	0.77	0.067
0.68	1.59	0.070	0.96	1.04	0.11	0.63	0.84	0.074
0.72	1.69	0.078				0.81	1.01	0.066
0.90	2.24	0.075				0.83	0.97	0.056

[a] ND, not determined.

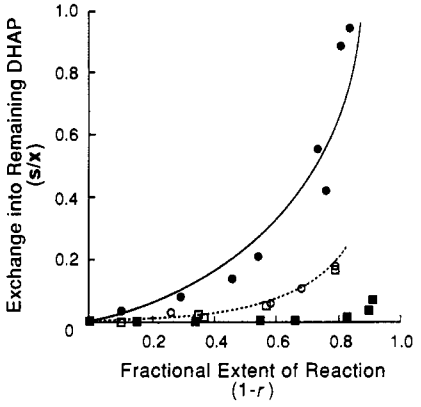

FIGURE 5: Incorporation of ^{3}H from tritiated water into remaining substrate dihydroxacetone phosphate as a function of the extent of reaction. The isotopic content (s/x) of the remaining substrate dihydroxyacetone phosphate is plotted as a function of conversion ($1 - r$) for the wild-type enzyme (●) and for the H95N (■), H95N,S96P (□), and E165D,S96P (○) mutant isomerases. The data for the wild-type enzyme are from Maister et al. (1976). The fitted line (solid) for the wild-type enzyme data is derived from the equation $s/x = A_7'(1 - r^{A_6'-1})/(A_6' - 1)$ (Albery & Knowles, 1976a), with $A_6' = 0.50$ and $A_7' = 0.27$ (Maister et al., 1976). No line is drawn for the data from the H95N enzyme. The fitted line (dotted) for the data from the H95N,S96P and E165D,S96P mutant enzymes is derived from the same equation, with $A_7' = 0.055$ and $A_6' = 0.15$.

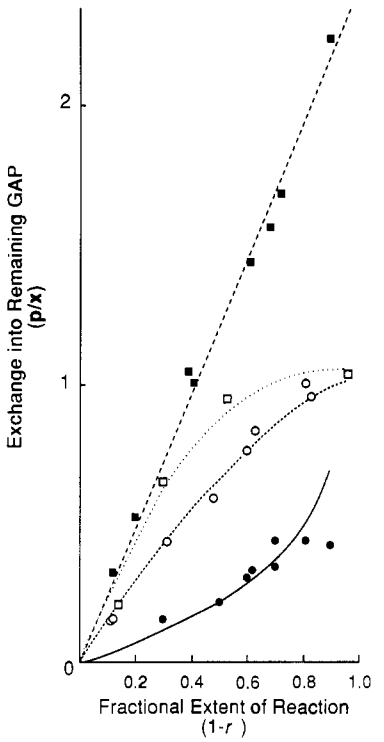

FIGURE 6: Incorporation of ^{3}H from tritiated water into remaining substrate glyceraldehyde 3-phosphate as a function of the extent of reaction. The isotopic content (p/x) of the remaining substrate glyceraldehyde 3-phosphate is plotted as a function of conversion ($1 - r$) for the wild-type enzyme (●) and for the H95N (■), H95N,S96P (□), and E165D,S96P (○) mutant isomerases. The data for the wild-type enzyme are from Fletcher et al. (1976). The fitted line (solid) for the wild-type enzyme data is derived from the equation $s/x = B_7'(1 - r^{B_6'-1})/(B_6' - 1)$ (Albery & Knowles, 1976a), with $B_6' = 1.14$ and $B_7' = 0.35$ (Fletcher et al., 1976). The fitted line (dotted) for the data from the H95N,S96P enzyme is derived from the same equation, with $B_6' = 3.4$ and $B_7 = 2.6$. The fitted line (dashed) for the E165D,S96P mutant enzyme has $B_6' = 2.3$ and $B_7' = 1.5$. The fitted line (heavy dashed) drawn for the H95N enzyme is a straight line of slope 2.3 passing through the origin.

product [1-^{14}C]dihydroxyacetone phosphate (s) relative to the specific radioactivity of solvent (x) is listed for several extents of reaction in Table IV.

Discussion

The energetics of the reaction catalyzed by several mutant triosephosphate isomerases have been defined, to gain a better understanding of the functional consequences of changes to amino acid residues in the active site. The primary changes of E165D (Straus et al., 1985) and H95N were introduced to probe the function of two of these residues in the catalyzed reaction. The E165D mutant was designed to assess the importance of the precise placement of the carboxylate base in the enolization steps (Figure 1), and the H95N mutation was made to complement H95Q in the analysis of how H95 contributes to catalysis by the isomerase.

The assignment of E165 as the enzymic base responsible for abstraction of the *pro-R* proton from DHAP or the C-2 proton from GAP seems secure. Affinity labeling work with glycidol phosphate and with bromohydroxyacetone phosphate (Hartman, 1968; Waley et al., 1970; de la Mare et al., 1972) showed that this glutamate residue is at the active site, and other lines of evidence suggested that it acts as the base in the catalyzed reaction (Plaut & Knowles, 1972; Herlihy et al., 1976). The high-resolution crystal structure of the yeast enzyme with the inhibitor phosphoglycolohydroxamate bound at the active site (Davenport, 1986) shows that E165 is exquisitely placed for the removal of the *pro-R* proton from a molecule of the substrate DHAP placed analogously. The E165D mutant, in which the enolization of DHAP has become rate limiting, has a k_{cat}/K_m value about 300-fold lower than that of the wild type (Raines et al., 1986), and this result confirms the expectation that the carboxylate base at the active site must be precisely oriented for efficient catalysis.

The electrophilic component of isomerase catalysis was originally probed by two methods. First, it was found that the rate of reduction of enzyme-bound DHAP by sodium

borohydride is nearly 10 times faster than the rate of reduction of free DHAP (Webb & Knowles, 1974, 1975). This rate acceleration suggested the possibility that the carbonyl group of DHAP becomes more polarized as it binds to the active site of the enzyme, and this view was confirmed when the carbonyl stretching frequency was examined by Fourier transform infrared spectroscopy (Belasco & Knowles, 1980). A shift of 19 cm^{-1} to lower frequency was observed for DHAP when it binds to the wild-type enzyme. The amino acid residues responsible for this facet of catalysis by the isomerase have been ascribed to H95 and K13 on the basis of the crystal structures of the chicken and yeast enzymes (see Figure 2). There has been considerable discussion, however, over the precise role of H95 (Alber et al., 1987; Nickbarg et al., 1988). Since the δ- and ϵ-nitrogen atoms of histidine may be similar in position to the amide nitrogens of asparagine and glutamine, respectively (Lowe et al., 1985), it was possible that a neutral imidazole group at H95, acting in the catalyzed reaction as a hydrogen-bond donor, could be effectively substituted by asparagine or glutamine. In fact, neither the H95Q nor the H95N enzyme performs near to the wild-type level. The H95Q mutant manages to catalyze the isomerization at about 1/400 of the wild-type level (when the values of k_{cat}/K_m are compared), but the mechanism of proton transfers is subtly altered in this enzyme (Nickbarg et al., 1988). The H95N mutant is even more sluggish as an isomerase, with the value of k_{cat}/K_m reduced by a factor of 3500 compared to that of the wild type (Table I). In addition, the H95N isomerase no longer forms DHAP exclusively from substrate GAP, and an alternate product, methylglyoxal, is produced by phosphate elimination about one-third of the time.

Generation of Pseudorevertants. Two of the site-directed mutants, E165D and H95N, were used as starting points in a search for enzymes with changes at second sites that would confer increases in specific catalytic activity (Hermes et al., 1987a,b). We embarked on this search both to learn about the ways in which enzymatic catalysis can be improved and to find out how readily a particular catalytic lesion might be overcome. The two isomerases E165D and H95N were therefore subjected to random mutagenesis (Hermes et al., 1987a,b). Chemical mutagenesis (Myers et al., 1985) of the gene encoding the E165D isomerase provided one pseudorevertant of improved catalytic activity: E165D,S96P. On treating the gene encoding the H95N mutant with the same array of chemical reagents, a single catalytic pseudorevertant was also found: H95N,S96P.

The discovery that the S96P substitution partially suppresses *each* of the parental lesions, E165D and H95N, was surprising, and we considered two explanations. First, the S96P change could act as a "global suppressor", partially compensating in some way for each of the changes to the active site residues in E165D and H95N, from which we had searched for pseudorevertants. Such a global suppressor might stand in loose analogy to the thermal stability suppressors of *Staphylococcal* nuclease described by Shortle and Lin (1985). Alternatively, the S96P change could simply reflect the existence of a mutational "hot spot" (Benzer, 1961; Coulondre et al., 1978) that arises from the particular two- or three-dimensional structure of the isomerase gene and the nature of the chemical reagents used in the protocol of Myers et al. (1985). To distinguish between these possibilities, an enzyme with the single substitution of serine-96 with proline (S96P) was made. If S96P were a global suppressor, a catalytic improvement would be found. (Or rather, since the wild-type enzyme is encounter controlled, the rate of the reaction catalyzed by S96P would be unchanged.) In terms of k_{cat}/K_m, however, the S96P change in the context of the wild-type enzyme is deleterious (see Table I). Although the S96P change seems generally to improve the binding of inhibitors (such as arsenate or phosphoglycolohydroxamate) and of the substrate and product (as gauged by the values of K_m), this mutation has very different effects upon the transition-state free energy in the mutants and the wild type. Thus the S96P change increases k_{cat}/K_m for the E165D and the H95N mutants by 60- and 20-fold, respectively, yet the same change leads to a 20-fold *decrease* in k_{cat}/K_m for the wild-type enzyme. The activities of the three mutant enzymes, E165D, H95N, and S96P, and of the two pseudorevertants, E165D,S96P and H95N,S96P, and their relationship to the wild type are illustrated in Figure 3. These relationships demonstrate the nonadditivity of the changes at positions 165 and 96 and at positions 95 and 96 and show that S96P is not a global suppressor. It seems possible, therefore, that S96P appears as a suppressor mutation for each of the parental mutant enzymes E165D and H95N because this region of the gene lies in a mutagenic hot spot.[3] We have recently developed techniques for truly random mutagenesis that avoid this problem (Hermes et al., 1989), and the results from these experiments will be discussed elsewhere.

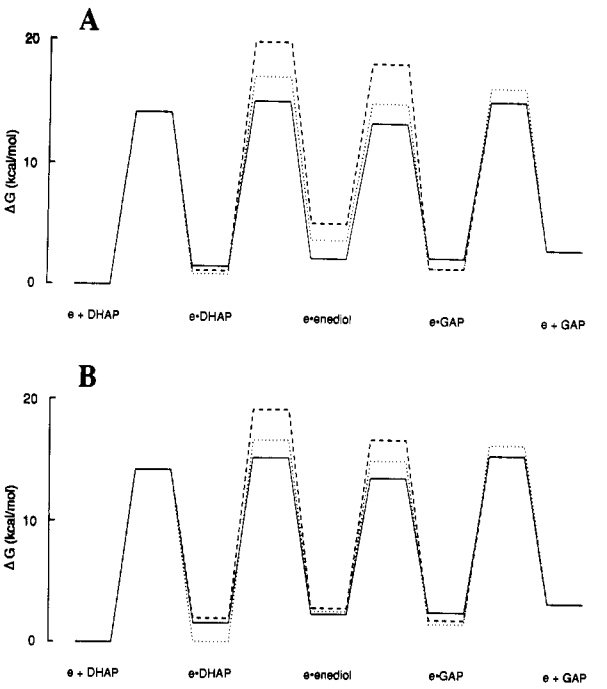

FIGURE 7: Free-energy profiles of mutant triosephosphate isomerases, constructed as described by Albery & Knowles (1976a,b). (A) For the reaction catalyzed by the wild-type (solid), the mutant H95N (dashed), and the pseudorevertant H95N,S96P (dotted) enzymes. The profile for the wild-type isomerase is from Albery and Knowles (1976b). (B) For the reaction catalyzed by the wild-type (solid), the slow mutant E165D (dashed), and the pseudorevertant E165D,S96P (dotted) enzymes. The profile for the wild-type isomerase is from Albery and Knowles (1976b).

Evaluation of the Reaction Energetics. To understand more completely the kinetic effects of these active site mutants and their second-site suppressors, free-energy profiles for the H95N enzyme and for the two pseudorevertants H95N,S96P and E165D,S96P have been obtained. [The energetics of E165D have been reported earlier (Raines et al., 1986).] The reaction

[3] Although we have not investigated the mutation frequency at the codon for S96, we consider it unlikely that the chemical mutagenesis protocol *is* random and that the S96P change happens uniquely to offset the catalytic damage inflicted by E165D or H95N.

energetics of these mutant isomerases were defined as described below, and the resulting free-energy profiles are plotted in Figure 7.

A tritium-*transfer* experiment measures the extent to which the proton that is abstracted from substrate ends up as the new carbon-bound proton of the product. With the wild-type enzyme, the amount of tritium transferred from the *pro-R* position at C-1 of substrate DHAP to C-2 of the product GAP was found to be about 6% after complete reaction (Herlihy et al., 1976); the rest of the substrate's tritium label was lost to solvent. For the three enzymes H95N, H95N,S96P, and E165D,S96P, proton loss from substrate DHAP to solvent is even more rapid (see the values of p/s_0 in Table II). Only rarely does a tritium find its way from C-1 of substrate DHAP to C-2 of product GAP. This behavior was also found for the E165D isomerase (Raines et al., 1986).

An *enrichment* experiment examines which step is rate limiting up to the formation of the intermediate enediol(ate). For the three mutant enzymes H95N, H95N,S96P, and E165D,S96P, the specific radioactivity of reisolated DHAP rises as the reaction proceeds (Figure 4). The isotopically sensitive enolization step (that is, the abstraction of the proton from DHAP) must therefore be partially rate limiting in the overall reaction.[4]

The *discrimination* against the appearance of solvent tritium in the product measures the extent to which the proton-transfer step is rate limiting in the collapse of the enediol(ate) intermediate and the release of product. When DHAP is the substrate for the wild-type isomerase, only a relatively small (1.3-fold) discrimination against tritium is observed in the formation of product GAP. For both pseudorevertant isomerases (E165D,S96P and H95N,S96P) there is also only a small discrimination against solvent tritium (of 1.04- and 1.5-fold, respectively) for the reaction in this direction. When the H95N enzyme is used, however, the specific radioactivity of the product GAP is much less than the specific radioactivity of the solvent, reflecting a 7-fold discrimination against tritium (see the values of p/x in Table III). From these results, we conclude that the step in which the enediol(ate) is protonated to form GAP is slower than the release of product GAP for the H95N isomerase, but for the H95N,S96P and E165D,S96P pseudorevertants, product release is slower than proton transfer.

When GAP is the substrate, all of the reactions show substantial discrimination against tritium in the formation of product DHAP, ranging from 10- to 15-fold (see the values of s/x in Table IV). these large values for discrimination against tritium parallel the outcome for the wild-type enzyme (for which a 9-fold discrimination is seen) and show that enolization is rate limiting in the formation of the enediol(ate) from DHAP. The high discriminations against tritium (10–15-fold) when GAP is the substrate for the H95N, H95N,S96P, and E165D,S96P mutants are consistent with the enrichment values for these mutants. The enrichment curves of Figure 4 show that the H95N, H95N,S96P, and E165D,S96P mutants behave almost identically, and these curves reflect the kinetic isotope effect on the rate-limiting enolization of DHAP to give the enediol(ate).

The *exchange-conversion* experiments provide information about how the enediol(ate) partitions between substrate and product and define the relative overall rates for the forward and reverse processes. In an exchange-conversion experiment, the isomerase-catalyzed reaction is run in tritiated water under irreversible conditions. For example, the reaction of unlabeled DHAP is followed to determine how the intermediate enediol partitions between reaction forward ("conversion") to give product GAP and reaction back ("exchange") to give tritiated substrate DHAP. The result for the wild-type enzyme is contrasted with the data for the mutant enzymes in Figure 5. For the wild-type enzyme, the slope of the line is about 0.3 at the start of the reaction, indicating that the enediol(ate) is converted to product (GAP) three times for each time it undergoes exchange and returns to substrate DHAP. For the H95N mutant, in contrast, essentially no radioactivity appears in the remaining substrate (Figure 5). The enediol(ate) partitions forward at least 30 times for each time it returns back to substrate. The energy barrier for the production of tritiated DHAP is higher than the barrier that leads to the formation of free product, GAP. The exchange-conversion plots when H95N,S96P and E165D,S96P are used are similar to that for H95N. For both of these pseudorevertants, H95N,S96P and E165D,S96P, the intermediate enediol partitions forward to product GAP about 20 times for each time it exchanges to give tritiated DHAP.

These conclusions are borne out by the exchange-conversion experiments carried out in the reverse direction, with GAP as the substrate. For the H95N mutant enzyme, the enediol(ate) partitions back to give tritiated GAP about 2.5 times as often as it is converted to product DHAP (see Figure 6). [Eventually, the specific radioactivity of the reisolated GAP exceeds the specific radioactivity of the solvent in which the reaction is run. This presumably occurs because the H95N enzyme also catalyzes the formation of methylglyoxal from GAP. The consumption of [^{3}H]GAP (which is formed in the exchange process) is discriminated against to an even greater extent in the side reaction, allowing the tritiated material to build up to a specific radioactivity greater than that of the solvent.] When the pseudorevertant enzyme E165D,S96P is used, the intermediate enediol(ate) partitions forward to DHAP twice for every three times it suffers exchange to give tritiated GAP (Figure 6). This ratio compares the rate of formation of DHAP from the enediol(ate) with the rate of production of tritiated GAP. Since there is only a small discrimination against tritium in the formation of GAP from the enediol with this mutant, the exchange:conversion ratio directly establishes the partitioning of the enediol(ate) intermediate between GAP and DHAP. When H95N,S96P is the enzyme, the enediol(ate) partitions forward twice for every five times it exchanges to give tritiated GAP. This experiment establishes the relative heights of the barriers for the H95N,S96P pseudorevertant. The information about the relative rates of individual steps in the reactions catalyzed by the mutant enzymes provided by the experiments described above can be combined with the steady-state kinetic results to construct free-energy profiles for each of these reactions. Although the method for deriving such free-energy profiles has been detailed earlier (Albery & Knowles, 1976a,b), several points relevant to the construction of the profiles for these mutant enzymes should be mentioned here. First, a standard-state concentration for the triose phosphate substrates of 40 μM, equal to the known triose phosphate levels in vivo (Williamson, 1965), was assumed. Second, for each mutant enzyme, the height of the free-energy barrier for the kinetically significant transition state is essentially determined by the value of k_{cat} for DHAP as substrate, since the enolization of DHAP is rate-limiting for each of these enzymes.[5] Third, for lack of a better model, the

[4] Strictly, if isotope exchange between the conjugate acid of the enzyme's base and the solvent is very rapid, then we may only conclude that the enolization of DHAP is slow relative to the steps that precede the formation of the intermediate.

dissociation constant for the intermediate analogue phosphoglycolohydroxamate relative to that of the wild-type enzyme was used to set the free energy of the enzyme-bound enediol(ate). The derived free-energy profiles are presented in Figure 7. Figure 7A contrasts the energetics of the mutant enzyme H95N with those of its pseudorevertant, H95N,S96P, and with those of the wild-type enzyme. Figure 7B compares the free-energy profile of E165D (Raines et al., 1986) with that of its pseudorevertant, E165D,S96P, and with that of the wild type enzyme.

Kinetic Consequences of Pseudoreversion. From these results, what can we say about the consequences of the second-site S96P change upon the two primary isomerase mutants E165D and H95N? First, for each of the E165D,S96P and H95N,S96P pseudorevertants, GAP release seems to be slowed about 5–10-fold with respect to the wild type. Looking at the reverse reaction (that is, the formation of the enzyme–GAP complex from GAP and the free enzyme), such a reduction in the rate of productive complex formation need not come as a surprise. Indeed, the rates of encounter of different substrates or inhibitors with the same enzyme are often seen to vary over an order of magnitude or more (Morrison & Walsh, 1987; Stein et al., 1987a,b; Bartlett & Marlowe, 1987; Duncan & Walsh, 1988; Schloss, 1988), and productive complexes of different enzymes (or point mutants of the same enzyme) with the same substrate need not always form at the same rate (Klapper et al., 1986; Sharp et al., 1987). It is possible that, following the formation of an encounter complex, a first-order rearrangement is required for the proper positioning of the substrate with respect to the enzyme's catalytic groups. In this case, we should not be surprised that the introduction of a prolyl residue (as in S96P) affects the rate at which productive complexes form, since this amino acid has stronger stereochemical constraints than any other residue (Richardson, 1981). It is also possible that the introduction of proline at position 96 interferes with the closing of the flexible loop, which is now known to be a critical element of catalysis by the isomerase (Pompliano et al., 1990).

In Figure 8, we illustrate the energetic consequences both of the primary mutations of H95N and E165D and of the partial suppression of each of these lesions that is effected by S96P. When compared to the wild-type enzyme, the E165D and H95N enzymes are much less effective at stabilizing the transition states for the catalytic (proton abstraction) steps. In contrast, the free energies of the enzyme–substrate and enzyme–product complexes are largely unaffected (see Figure 8A). Introduction of the S96P change counteracts this transition-state destabilization in both cases (see Figure 8B). Thus, for the H95N,S96P pseudorevertant, both enolization steps are speeded up by about 50-fold, while the K_m value for each of the substrates remains about the same as in the H95N parent. Two observations suggest that the intermediate enediol(ate) is also bound more tightly to the pseudorevertant than to H95N. First, the intermediate analogue phosphoglycolohydroxamate (Collins, 1974) is a 10-fold better inhibitor of the pseudorevertant enzyme than of H95N. Second, unlike its H95N parent which produces methylglyoxal one-third of the time from GAP as substrate, the H95N,S96P pseudorevertant converts GAP entirely into product DHAP, and no detectable amount of the enediol(ate) intermediate is released into solution to yield methylglyoxal.

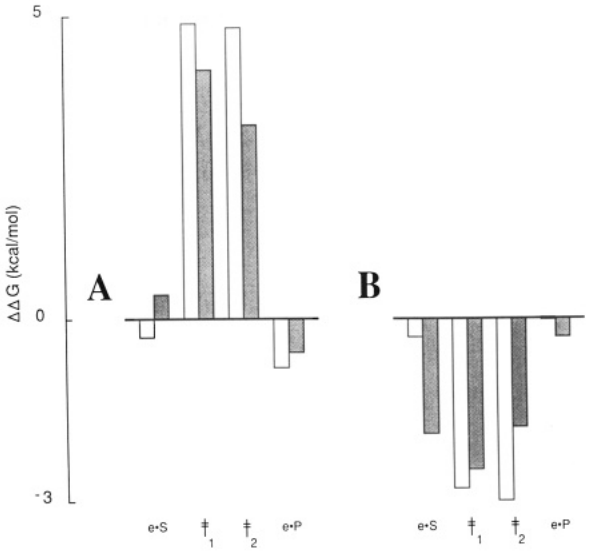

FIGURE 8: Free-energy changes for the two pseudorevertant enzymes, relative to their parental mutants. (A) Open bars: difference in the free energy ($\Delta\Delta G$) for each state of the reaction catalyzed by H95N, relative to the wild-type enzyme. Shaded bars: difference in the free energy for each state of the reaction catalyzed by E165D, relative to the wild-type enzyme. (B) Open bars: difference in the free energy for each state of the reaction catalyzed by H95N,S96P, relative to H95N. Shaded bars: difference in the free energy for each state of the reaction catalyzed by E165D,S96P, relative to E165D. The enzyme–dihydroxyacetone phosphate complex is labeled e·S, and the enzyme–glyceraldehyde 3-phosphate complex is labeled e·P. The transition states for the enolization of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are denoted by the symbols $‡_1$ and $‡_2$, respectively.

The effects of the S96P change on the energetics of the reaction catalyzed by the E165D enzyme are surprisingly similar to those described for the S96P pseudorevertant from H95N. The transition state for each proton abstraction step is stabilized considerably in the E165D,S96P pseudorevertant when compared to the E165D enzyme (Figure 8B). Unlike the H95N,S96P pseudorevertant, however, the E165D,S96P enzyme forms a tighter enzyme–DHAP complex than does its parent, and as a result the value of k_{cat} for DHAP as substrate is about the same for the E165D enzyme and for its pseudorevertant. As is evident from Figure 8B, the pseudorevertant enzyme has in each case partially corrected the primary catalytic lesion. Furthermore, although S96P compensates for a change in an active site electrophile in one case and for a movement of the active site base in the other, a remarkably similar pattern of transition-state stabilization results from the introduction of S96P into the two different enzymes H95N and E165D.

How might S96P be exerting its effects on catalysis? Starting from H95N, the adjacent S96P change may reorient asparagine-95 so that it is better positioned to form hydrogen bonds to the intermediate enediol(ate) and the flanking transition states. When S96P compensates for the E165D change, the binding of the intermediate, its adjacent transition states, and substrate DHAP are all improved. In contrast, when the S96P change is made in the wild-type sequence, an enzyme very similar to the E165D,S96P enzyme is produced. Detailed structural results will be needed to interpret these interesting relationships fully.

The analysis of the kinetics of several mutant isomerases in this work has expanded our understanding of the structure–function relationship in enzymatic catalysis. It is noteworthy that several different sets of active site residues are

[5] For both of the pseudorevertant enzymes (H95N,S96P and E165D,S96P) the rate of release of GAP from the enzyme·GAP complex is slower than the conversion of the enediol(ate) intermediate into GAP, and the free energy barrier shown for this enolization step is thus an upper limit.

capable of catalyzing the triosephosphate isomerization at an appreciable rate. Our experiments suggest that, even within a specific tertiary fold, there are several different ways in which substantial catalytic function can be achieved and that compensatory changes for deleterious mutations are not vanishingly rare.

ACKNOWLEDGMENTS

We thank Jeff Hermes for helpful discussions in the early phases of this work.

REFERENCES

Alber, T. C., Davenport, R. C., Giammona, D. A., Lolis, E., Petsko, G. A., & Ringe, D. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 603–613.

Albery, W. J., & Knowles, J. R. (1976a) *Biochemistry 15*, 5588–5600.

Albery, W. J., & Knowles, J. R. (1976b) *Biochemistry 15*, 5627–5631.

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.

Babul, J. (1978) *J. Biol. Chem. 253*, 4350–4355.

Ballou, C. E. (1960) *Biochem. Prep. 7*, 45–50.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D., & Waley, S. G. (1975) *Nature (London) 255*, 609–614.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., & Wilson, I. A. (1976) *Biochem. Biophys. Res. Commun. 2*, 146–155.

Bartlett, P. K., & Marlowe, C. K. (1987) *Biochemistry 26*, 8553–8561.

Belasco, J. G., & Knowles, J. R. (1980) *Biochemistry 19*, 472–477.

Benzer, S. (1961) *Proc. Natl. Acad. Sci. U.S.A. 43*, 403–415.

Blacklow, S. C., Raines, R. T., Lim, W. A., Zamore, P. D., & Knowles, J. R. (1988) *Biochemistry 27*, 1158–1167.

Bloom, B., & Topper, Y. J. (1956) *Science 124*, 982–983.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Collins, K. D. (1974) *J. Biol. Chem. 249*, 136–142.

Coulondre, C., Miller, J. H., Farabaugh, P. J., & Gilbert, W. (1978) *Nature (London) 274*, 775–780.

Davenport, R. C., Jr. (1986) Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.

de la Mare, S., Coulson, A. F. W., Knowles, J. R., Priddle, J. D., & Offord, R. E. (1972) *Biochem. J. 129*, 321–331.

Duncan, K., & Walsh, C. T. (1988) *Biochemistry 27*, 3709–3714.

Fletcher, S. J., Herlihy, J. M., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5612–5617.

Gerlt, J. A. (1987) *Chem. Rev. 87*, 1079–1105.

Guilford, W. J., Copley, S. C., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5013–5019.

Hartman, F. C. (1968) *Biochem. Biophys. Res. Commun. 33*, 888–894.

Herlihy, J. M., Maister, S. G., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5601–5607.

Hermes, J. D., Blacklow, S. C., & Knowles, J. R. (1987a) *Cold Spring Harbor Symp. Quant. Biol. 52*, 597–602.

Hermes, J. D., Blacklow, S. C., Gallo, K. A., Bauer, A. J., & Knowles, J. R. (1987b) in *Protein Structure, Folding, and Design 2*, pp 257–264, A. R. Liss, New York.

Hermes, J. D., Parekh, S. M., Blacklow, S. C., Köster, H., & Knowles, J. R. (1989) *Gene 84*, 143–151.

Horecker, B. L., & Kornberg, A. (1948) *J. Biol. Chem. 175*, 385–390.

Howell, E. H., Warren, M. S., Booth, C. L. J., Villafranca, J. E., & Kraut, J. (1987) *Biochemistry 26*, 8591–8598.

Klapper, I., Hagstrom, R., Fine, R., Sharp, K., & Honig, B. (1986) *Proteins 1*, 47–59.

Knowles, J. R. (1987) *Science 236*, 1252–1258.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Leatherbarrow, R. J., & Fersht, A. R. (1986) *Protein Eng. 1*, 7–16.

Lowe, D. M., Fersht, A., & Wilkinson, A. J. (1985) *Biochemistry 24*, 5106–5109.

Maister, S. G., Pett, C. P., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5607–5612.

Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Morrison, J. F., & Walsh, C. T. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 57*, 201–301.

Myers, R. M., Lerman, L. S., & Maniatis, T. (1985) *Science 229*, 242–247.

Nickbarg, E. B., Davenport, R. C., Petsko, G. A., & Knowles, J. R. (1988) *Biochemistry 27*, 5948–5960.

Plaut, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 311–320.

Pompliano, D. L., Peyman, A., & Knowles, J. R. (1990) *Biochemistry* (in press).

Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 301–310.

Racker, E. (1957) *Methods Enzymol. 3*, 293–296.

Raines, R. T., Sutton, E. L., Straus, D. R., Gilbert, W., & Knowles, J. R. (1986) *Biochemistry 25*, 7142–7154.

Richardson, J. (1981) *Adv. Protein Chem. 34*, 167–339.

Rieder, S. V., & Rose, I. A. (1956) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 15*, 337.

Rieder, S. V., & Rose, I. A. (1959) *J. Biol. Chem. 234*, 1007–1010.

Schloss, J. V. (1988) *Acc. Chem. Res. 21*, 348–353.

Sharp, K., Fine, R., & Honig, B. (1987) *Science 236*, 1460–1463.

Shaw, W. V. (1987) *Biochem. J. 246*, 1–17.

Shortle, D., & Lin, B. (1985) *Genetics 110*, 539–555.

Stein, R. L., Strimpler, A. M., Edwards, P. D., Lewis, J. J., Mauger, R. C., Schwartz, J. A., Stein, M. M., Trainor, D. A., Wildonger, R. A., & Zottola, M. A. (1987a) *Biochemistry 26*, 2682–2689.

Stein, R. L., Strimpler, A. M., Viscarello, B. R., Wildonger, R. A., Mauger, R. C., & Trainor, D. A. (1987b) *Biochemistry 26*, 4126–4130.

Straus, D., & Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2014–2018.

Straus, D., Raines, R. T., Kawashima, E., Knowles, J. R., & Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2272–2276.

Waley, S. G., Miller, J. C., Rose, I. A., & O'Connell, E. L. (1970) *Nature (London) 227*, 181.

Webb, M. R., & Knowles, J. R. (1974) *Biochem. J. 141*, 589–592.

Webb, M. R., & Knowles, J. R. (1975) *Biochemistry 14*, 4692–4698.

Williamson, J. R. (1965) *J. Biol. Chem. 240*, 2308–2321.