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Triosephosphate Isomerase: Energetics of the Reaction Catalyzed by the Yeast Enzyme Expressed in *Escherichia coli*†

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ABSTRACT: Triosephosphate isomerase from bakers' yeast, expressed in *Escherichia coli* strain DF502(p12), has been purified to homogeneity. The kinetics of the reaction in each direction have been determined at pH 7.5 and 30 °C. Deuterium substitution at the C-2 position of substrate (*R*)-glyceraldehyde phosphate and at the 1-*pro-R* position of substrate dihydroxyacetone phosphate results in kinetic isotope effects on k_{cat} of 1.6 and 3.4, respectively. The extent of transfer of tritium from [1(*R*)-^{3}H]dihydroxyacetone phosphate to product (*R*)-glyceraldehyde phosphate during the catalyzed reaction is only 3% after 66% conversion to product, indicating that the enzymic base that mediates proton transfer is in rapid exchange with solvent protons. When the isomerase-catalyzed reaction is run in tritiated water in each direction, radioactivity is incorporated both into the remaining substrate and into the product. In the "exchange-conversion" experiment with dihydroxyacetone phosphate as substrate, the specific radioactivity of remaining dihydroxyacetone phosphate rises as a function of the extent of reaction with a slope of about 0.3, while the specific radioactivity of the product is 54% that of the solvent. In the reverse direction with (*R*)-glyceraldehyde phosphate as substrate, the specific radioactivity of the product formed is only 11% that of the solvent, while the radioactivity incorporated into the remaining substrate (*R*)-glyceraldehyde phosphate also rises as a function of the extent of reaction with a slope of 0.3. These results have been analyzed according to the protocol described earlier to yield the free energy profile of the reaction catalyzed by the yeast isomerase. The profile shows that the isomerase from yeast, like the enzyme from chicken muscle previously analyzed, is a highly efficient catalyst. The kinetic characteristics of the yeast enzyme are very close to those of the chicken isomerase and provide the necessary basis with which the behavior of mutant yeast isomerases can be compared.

To understand the relationship between structure and function in enzymology and to evaluate the role of particular amino acid side chains, it is common practice to observe the consequences of changing catalytic residues by chemical modification or by site-directed mutagenesis. To maximize our understanding, we prefer to use an enzyme system that is already well characterized in structural, mechanistic, and energetic terms. One such system is triosephosphate isomerase (TIM, EC 5.3.1.1), a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and (*R*)-glyceraldehyde phosphate.[1] The rate of the enzymatic reaction is more than 10^9 times faster than that of the analogous reaction catalyzed by acetate ion (Hall & Knowles, 1975; Richard, 1984), and the reaction proceeds by the stereospecific abstraction of either the 1-*pro-R* proton of dihydroxyacetone phosphate or the C-2 proton of (*R*)-glyceraldehyde phosphate by an enzymic base to give a *cis*-enediol intermediate (Figure 1) (Rieder & Rose, 1959; Rose, 1962). The free energy profile of the reaction catalyzed by the isomerase from chicken muscle has been determined from a series of isotopic experiments (Albery & Knowles, 1976b) and shows that this enzyme is near to being optimal in catalytic terms (Albery & Knowles, 1976c, 1977; Knowles & Albery, 1977). Given the levels of triose phosphates in vivo and assuming that the substrates and the enzyme diffuse freely, it appears that the catalytic flux per enzyme molecule could not be increased by further modification of the protein.

The amino acid sequences of triosephosphate isomerases have been determined from a variety of organisms, both prokaryotic (Artavanis-Tsakonis & Harris, 1980; Pichersky et al., 1984) and eukaryotic (Kolb et al., 1974; Corran & Waley, 1975; Alber & Kawasaki, 1982; Lu et al., 1984; Maquat et al., 1985; Russell, 1985; Straus & Gilbert, 1985; McKnight et al., 1986; Marchionni & Gilbert, 1986; Swinkels et al., 1986). The isomerase from chicken muscle has been crystallized, and the structure has been determined to 2.5-Å resolution (Banner et al., 1975). The structure of the triosephosphate isomerase from yeast has been solved at 3-Å

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[1] Nomenclature: dihydroxyacetone phosphate, dihydroxyacetone 3-phosphate; glyceraldehyde phosphate, (*R*)-glyceraldehyde 3-phosphate (otherwise D-glyceraldehyde 3-phosphate); glycerol phosphate, (*R*)-glycerol phosphate (otherwise *sn*-glycerol phosphate); phosphoglycerate, 3-phospho-(*R*)-glycerate (otherwise 3-phospho-D-glycerate).

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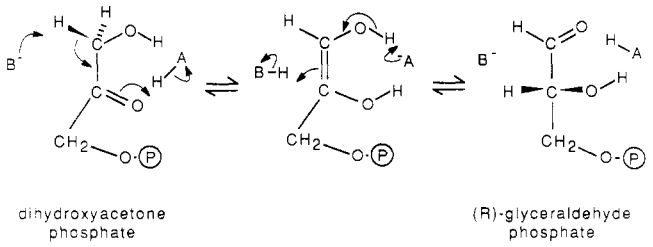

FIGURE 1: Reaction catalyzed by yeast triosephosphate isomerase. B^- is the enzymic base (Glu-165), and HA is an enzymic acid (His-95, or possibly Lys-12).

resolution (Alber et al., 1981), and the structures of an enzyme–substrate complex (Alber et al., 1981) and of an enzyme–inhibitor complex (R. Davenport, B. Seaton, G. Petsko, and D. Ringe, unpublished results) have been determined with the enzyme from this source. Although the amino acid sequences of the chicken and yeast isomerases show only about 50% similarity, the catalytically important residues appear all to be conserved (Alber & Kawasaki, 1982). Despite differences in the space groups of the crystals used, the chicken and yeast enzymes are strikingly alike in their overall three-dimensional structure (Alber et al., 1981).

The triosephosphate isomerase from bakers' yeast has previously been cloned and expressed (Casal et al., 1987) in *Escherichia coli* DF502, a strain from which the endogenous *E. coli* isomerase gene has been excised. We report here the isolation and purification of the yeast enzyme from this source and the determination of the free energy profile of the catalyzed reaction. We compare this profile with that previously determined for the chicken muscle enzyme, and (in the following paper) with that of a yeast isomerase mutant from which a putatively electrophilic catalytic group has been removed.

Experimental Procedures

Materials

E. coli strain DF502(p12) was a gift from R. C. Davenport and G. A. Petsko. DF-502, a streptomycin-resistant strain of *E. coli* that lacks the endogeneous *E. coli* isomerase [constructed in the same manner as DF500 by D. Fraenkel (Babul, 1978)], was a gift from D. Fraenkel, and the gene encoding the yeast isomerase (Alber & Kawasaki, 1982) was expressed by use of a pUC18 vector under control of the *lac* operon. The details of the construction and expression of the yeast isomerase in *E. coli* are given in Casal et al. (1987).

Native chicken triosephosphate isomerase was purified by J. G. Belasco according to Putnam 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 Sigma Chemical Co. (St. Louis, MO). Glycerophosphate dehydrogenase (rabbit muscle) and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were initially obtained from Sigma. Later experiments used the dehydrogenases from Boehringer-Mannheim (Indianapolis, IN) and Serva (Westbury, NY), respectively. 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), 2,3-diphospho-(*R*)-glycerate [pentakis(cyclohexylammonium) salt], NAD^+, NADH (disodium salt), ATP (disodium salt, grade 1), (*RS*)-glycerol phosphate (disodium salt, hexahydrate), 2-phosphoglycolate [tris(monocyclohexylammonium) salt], Dowex-50W (H^+ form, 100–200 mesh, 4% cross-linked), and QAE-Sephadex A-50 (Cl^- form) were from Sigma.[2] 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). 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 according to the method of Herlihy et al. (1976). ^2H_2O (99.8% ^2H) was from Merck (Rahway, NJ), and tritiated water (4.5 Ci/mL) was from Amersham (Chicago, IL). Scintiverse-II scintillation cocktail was from Fisher (Medford, MA). Bacto tryptone and Bacto yeast extract were from Difco Labs (Detroit, MI). Ammonium sulfate was of special enzyme grade from Schwarz/Mann (Cambridge, MA). All other chemicals and reagents were of the best available commercial grades.

Methods

Rich medium was LB (Miller, 1972) supplemented with (in 1 L) ampicillin (sodium salt, 150 mg), uracil (40 mg), histidine (40 mg), and glycerol (2 mL). All media were prepared in distilled water. Samples (5–1000 μL) for radiochemical analysis were dissolved in scintillation cocktail (6–12 mL) and counted in a Beckman LS1801 automatic liquid scintillation counter. pH was measured with a Radiometer RHM62 pH meter fitted with a Radiometer GK2320 electrode and calibrated at room temperature with Fisher standard buffers. Conductivities were measured on a Radiometer CDM3 conductivity meter. Ultraviolet absorbance was measured on a Perkin-Elmer 554 spectrophotometer. An extinction coefficient for NADH at 340 nm of 6220 M^{-1} cm^{-1} was assumed (Horecker & Kornberg, 1948). All enzymatic reactions were conducted at 30 °C, unless noted otherwise. The concentration of purified yeast triosephosphate isomerase was measured by its absorbance at 280 nm, assuming that $A_{280}^{1\%} = 10$ (Norton & Hartman, 1972). 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 gels were fixed in aqueous ethanol (25% v/v) containing acetic acid (8% v/v) and were stained for protein with Coomassie blue. The molecular weight standards (Sigma) were α-lactalbumin (14 200), trypsinogen (24 000), carbonic anhydrase (29 000), ovalbumin (45 000), and bovine serum albumin (66 000). Protein assays were conducted either by the method of Lowry et al. (1951) using bovine serum albumin as standard or by the method of Bradford (1976) using purified yeast triosephosphate isomerase as the standard. Protein solutions were concentrated by ultrafiltration with Amicon PM-10 membranes (M_r 10 000) or by centrifugation (5000*g*) in an Amicon Centricon-10 ultrafiltration apparatus.

Triosephosphate isomerase activity was measured in 66 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (3.3 mM), according to Plaut and Knowles (1972). One unit of isomerase activity is that amount of enzyme re-

[2] 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.

quired to convert 1 μmol of (*R*)-glyceraldehyde 3-phosphate to product in 1 min at 30 °C. For the determination of K_m, the data were fitted by the nonlinear least-squares program HYPERO (Cleland, 1979). A value of 26 500 for the isomerase subunit molecular weight was assumed (Krietsch et al., 1970).

Purification of Yeast Wild-Type Triosephosphate Isomerase from E. coli DF502(p12). Cell paste (9.7 g) of *E. coli* DF502(p12) obtained from 4.5 L of rich medium grown to A_{550nm} = 3.0 in 8 h was suspended in 25 mL of 10 mM Tris-HCl buffer, pH 7.6, containing EDTA (1 mM) and β-mercaptoethanol (1 mM) and lysed by passage through a French press cell at 12 000 psi and 4 °C. Cell debris was removed by centrifugation at 30000*g* for 1 h. All subsequent purification steps were conducted at 4 °C. To the supernatant (30 mL) was slowly added finely ground solid ammonium sulfate (12.6 g). The solution was equilibrated by stirring overnight. The precipitate was then removed by centrifugation at 30000*g* for 30 min, and the supernatant (31 mL) was slowly brought to 90% saturation by the addition of solid ammonium sulfate (5.9 g). After equilibration and centrifugation at 30000*g* for 30 min, the resulting pellet was resuspended in 20 mL of 10 mM Tris-HCl buffer, pH 7.6, containing EDTA (1 mM) and β-mercaptoethanol (1 mM) and dialyzed exhaustively against the same buffer. The sample was then loaded onto a column (20 cm × 1.5 cm²) of QAE-Sephadex A-50 that had been equilibrated with 10 mM Tris-HCl buffer, pH 7.6, containing EDTA (1 mM) and β-mercaptoethanol (1 mM) and eluted with a linear gradient (0–100 mM, 100 + 100 mL) of KCl in the same buffer. Fractions (2 mL) were collected and assayed for enzyme activity. Peak fractions were further analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Fractions containing isomerase activity were pooled, concentrated by ultrafiltration, and dialyzed overnight against 1 L of 10 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (1 mM) and β-mercaptoethanol (1 mM). The protein sample was diluted to a volume of 10 mL with distilled water, adjusted to pH 8.0 with 1 N NaOH, and loaded onto a column (20 cm × 1.77 cm²) of QAE-Sephadex A-50 equilibrated with 10 mM triethanolamine hydrochloride buffer, pH 8.0, containing EDTA (1 mM) and β-mercaptoethanol (1 mM). The column was eluted with a linear gradient (0–75 mM, 100 + 100 mL) of KCl in the same buffer. Fractions (2 mL) were analyzed as before, and those containing isomerase were pooled, dialyzed against 500 mL of 100 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (1 mM) and β-mercaptoethanol (1 mM), and concentrated by ultrafiltration. Chromatography on QAE-Sephadex at pH 8.0 was then repeated under the same conditions. The results of the purification procedure are shown in Table I.

[1(R)-²H]Dihydroxyacetone phosphate was prepared by equilibration of dihydroxyacetone phosphate with ^2H_2O and chicken triosephosphate isomerase as described by Leadlay et al. (1976), except that tritiated water (5.6 mCi/mL) was added to serve as a check on the completeness of isotopic exchange with the medium. The reaction was stopped by adjusting the pH to 2 with ^2HCl (12 N in ^2H_2O), and the solvent was removed by bulb-to-bulb distillation. The isomerase was then removed by washing the residue through a small column (3 cm × 0.25 cm²) of Dowex-50W (H^+ form) with distilled water (4 mL). The washings were adjusted to pH 7 with anhydrous K_2CO_3, and isomerase-free aldolase (2 units) was added to convert the equilibrium proportion of glyceraldehyde phosphate to fructose 1,6-bisphosphate. After incubation for 3 h at room temperature, aldolase was removed by Dowex-50W chromatography as above. The mixture of dihydroxyacetone phosphate and fructose 1,6-bisphosphate was then separated chromatographically on DEAE-cellulose. The sample was diluted with 10 mM triethylammonium bicarbonate buffer, pH 7.5 (150 mL), and distilled deionized water (150 mL). The sample (conductivity <1 mS) was then applied to a column (12 cm × 2.56 cm²) of DE-52 that had been equilibrated with 30 mM triethylammonium bicarbonate buffer, pH 7.5. The column was washed with 30 mM triethylammonium bicarbonate buffer, pH 7.5 (40 mL), and eluted with a linear gradient of the same buffer (30–300 mM, 200 + 200 mL) at 4 °C. Fractions (5 mL) were collected and assayed for radioactivity and dihdyroxyacetone phosphate concentration. Those fractions (fractions 22–28) that contained dihydroxyacetone phosphate were combined; the solution was adjusted to pH 4.5 with HCl (5 N) and then concentrated by evaporation under reduced pressure. The yield of labeled dihydroxyacetone phosphate was 38 μmol (45%). The specific radioactivity of the [1(*R*)-²H,³H]dihydroxyacetone phosphate (0.052 μCi/μmol) matched that of the solvent determined at the start of the synthesis. The sample was free of glyceraldehyde phosphate and of fructose bisphosphate. Nondeuteriated dihydroxyacetone phosphate for the measurement of the kinetic isotope effect was carried through the same purification procedure, analogously to the labeled material.

3-Phospho[2-²H]glycerate was prepared as described by Leadlay et al. (1976). The sample was purified by chromatography on DEAE-cellulose as described above. The specific radioactivity of 3-phospho[2-²H,³H]glycerate was 13.1 ± 0.2 μCi/mmol, which compared well with the specific radioactivity of the solvent at the start of the reaction, 12.3 ± 0.6 μCi/mmol.

[2-²H]Glyceraldehyde Phosphate. Labeled 3-phospho[2-²H]glycerate, containing a trace amount of the 2-³H material, was converted to [2-²H]glyceraldehyde phosphate with phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase, essentially as described by Leadlay et al. (1976), except that the reaction mixture contained 500 mM potassium formate buffer, pH 7.0, and magnesium acetate (500 mM), in place of triethanolamine hydrochloride buffer, and magnesium chloride. During the course of the reaction, the pH was periodically readjusted to neutrality with formic acid (1 N). After 90 min, cold distilled deionized water (700 mL) was added, and the pH was adjusted to pH 3.1 with formic acid (1 N). The mixture was then loaded onto a column (12 cm × 1.77 cm²) of AG1 ($HCOO^-$ form) that had been equilibrated with 25 mM triethylammonium formate buffer, pH 3.1. The column was eluted with a linear gradient of triethylammonium formate buffer (25 mM to 2 M, 200 + 200 mL). Fractions containing (*R*)-glyceraldehyde phosphate were combined and adjusted to pH 2.5 with freshly washed Dowex 50W (H^+ form) (4 g). The resin was then removed by centrifugation (6000*g* for 2 min). The pellet was resuspended twice in water (5 mL) and recentrifuged as above after each wash. The combined supernatants were concentrated to 3.4 mL by evaporation under reduced pressure. The specific radioactivity of the glyceraldehyde phosphate was 12.5 μCi/mmol, in good agreement with the specific radioactivity of the solvent used in the synthesis of the phosphoglycerate (12.3 μCi/mmol). Nondeuteriated (*R*)-glyceraldehyde phosphate for comparison in kinetic experiments was prepared from commercial phosphoglycerate in an exactly analogous manner to the deuteriated material.

Inhibition Studies. (*RS*)-Glycerol phosphate was assayed according to Fletcher et al. (1976). For the determination of

the K_i, five different concentrations of substrate in both the absence and presence of (*RS*)-glycerol phosphate (0.98 mM) were used. The kinetic constants were calculated with the nonlinear least-squares program COMPO (Cleland, 1979). Phosphoglycolohydroxamate and 2-phosphoglycolate were assayed by treatment with alkaline phosphatase followed by measurement of the inorganic phosphate released by the method of Chen and Toribara (1956). The K_i values were determined as described for (*RS*)-glycerol phosphate, with a concentration of the hydroxamate of 31.4 μM and of 2-phosphoglycolate of 167 μM.

Tritium Washout from [1(R)-^{3}H]Dihydroxyacetone Phosphate. The isomerization of stereospecifically labeled [1(*R*)-^{3}H]dihydroxyacetone phosphate catalyzed by yeast isomerase was studied according to the method of Herlihy et al. (1976) as modified by Raines et al. (1986). The reaction was started by the addition of a sample of the yeast isomerase and stopped at the desired point by lowering the pH to below 2 by the addition of HCl and rapid freezing in liquid N_2. Yeast isomerase is completely inactive under these conditions.

Appearance of Solvent Tritium in Remaining Substrate Dihydroxyacetone Phosphate and in Product. Isomerase-catalyzed reactions were run as described by Maister et al. (1976) as modified by Raines et al. (1986). Reaction mixtures were quenched by acidification as described above. The location of the ^{3}H label in the 3-phospho[2-^{3}H]glycerate was checked by treatment with phosphoglycerate mutase and enolase as described in Herlihy et al. (1976).

Appearance of Solvent Tritium in Remaining Substrate Glyceraldehyde Phosphate and in Product. The reaction was conducted according to Fletcher et al. (1976) as modified by Raines et al. (1986). Reaction mixtures were quenched by acidification as described above.

Determination of Specific Radioactivity. Specific radioactivity determinations of phosphoglycerate and glycerol phosphate were made directly on samples from column fractions as described by Raines et al. (1986). The results of all tritium experiments are described with the nomenclature of Albery and Knowles (1976a). The specific radioactivity of glycerol phosphate (or of its progenitor, dihydroxyacetone phosphate) is *s*, the specific radioactivity of the phosphoglycerate (or of its progenitor, glyceraldehyde phosphate) is *p*, and the specific radioactivity of the solvent is *x*.

Reactions of [1(R)-^{2}H]Dihydroxyacetone Phosphate and of [2-^{2}H]Glyceraldehyde Phosphate. Kinetic experiments using ^{2}H-labeled substrates were done as described in Leadlay et al. (1976). Assays with nondeuteriated substrates were done concurrently with those using deuteriated substrates to provide a reliable kinetic comparison. The values of K_m, V_{max}, and (k_{cat}^H/k_{cat}^D) were calculated with the nonlinear least-squares fitting program ISOVMVC (Cleland, 1979).

RESULTS

The purification of wild-type yeast triosephosphate isomerase expressed in *E. coli* strain DF502(p12) is summarized in Table I. The purified protein gave a single band both on polyacrylamide gels in sodium dodecyl sulfate and on nondenaturing isoelectric focussing gels, as shown in the following paper (Nickbarg et al., 1988). Protein sequence analysis of the N-terminal residues of the purified isomerase gave the expected sequence (Ala-Arg-Thr-Phe-). No N-terminal methionine was detected. The molecular weight of the yeast isomerase expressed in *E. coli* strain DF502(p12) was identical with that of a sample of commercial yeast isomerase, as judged by polyacrylamide gel electrophoresis under denaturing conditions.

Table I: Purification of Yeast Triosephosphate Isomerase from *E. coli* Strain DF502(p12)

step	total units	specific catalytic activity (units/mg)	*x*-fold purification	yield (%)
crude lysate	565 000	630		
ammonium sulfate pellet	115 000	2820	4.5	20
QAE-Sephadex (pH 7.5)	103 000	5600	9.0	18
QAE-Sephadex (pH 8.0)	59 200	14 900	24	10
QAE-Sephadex (pH 8.0)	15 090	19 250	31	3

Table II: Steady-State Parameters for Bakers' Yeast Triosephosphate Isomerase Expressed in *E. coli* DF502(p12) and for the Isomerase from Brewers' Yeast[a]

parameter[b]	enzyme from *E. coli* DF502(p12)[c]	enzyme from brewers' yeast[d]	units
k_{cat}^-	(8.7 ± 0.3) × 10^3	4.4 × 10^3	s^{-1}
K_m^-	1.5 ± 0.1	1.27 ± 0.06	mM
K_m^-(unhydrated)[e]	0.055 ± 0.004	0.048 ± 0.002	mM
k_{cat}^+	(7.5 ± 0.2) × 10^2	4.08 × 10^2	s^{-1}
K_m^+	2.3 ± 0.2	1.23 ± 0.06	mM
K_m^+(unhydrated)[e]	1.4 ± 0.1	0.73 ± 0.04	mM
$K_i(HAsO_4^{2-})$	9.6 ± 0.3	12 ± 2	mM
K_i((*RS*)-glycerol phosphate)[f]	1.4 ± 0.3		mM
K_i(phosphoglycolohydroxamate)[g]	15 ± 3		μM
K_i(2-phosphoglycolate)[h]	30 ± 1		μM
K_{eq}^i	18 ± 5	19	mM

[a] Data for the brewers' yeast enzyme are from Krietsch et al. (1970). [b] A (−) as superscript indicates (*R*)-glyceraldehyde 3-phosphate as substrate. A (+) as superscript indicates dihydroxyacetone phosphate as substrate. [c] All experiments were conducted at 30 °C in 66 mM triethanolamine hydrochloride buffer, pH 7.5, containing EDTA (3.3 mM). [d] The literature values for the brewers' yeast isomerase were determined at 25 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.6. [e] Values for the unhydrated forms of the substrates; see Albery and Knowles (1976b). [f] The value for the chicken enzyme is 0.84 mM (Jones, 1972). [g] The value for the chicken enzyme is 7.1 μM (Raines et al., 1986). [h] The value for the chicken enzyme is 6.8 μM (Johnson & Wolfenden, 1970). [i] The overall equilibrium constant as determined from the Haldane relationship.

The steady-state kinetic parameters for the yeast wild-type isomerase expressed in *E. coli* are listed in Table II. The value of k_{cat} for the enzyme, using glyceraldehyde 3-phosphate as substrate, was found to be constant between pH 6.5 and pH 8.4. All subsequent experiments with the wild-type isomerase were therefore conducted at pH 7.5 and 30 °C. The kinetic data for the bakers' yeast enzyme expressed in *E. coli* are in reasonable agreement with those for the native isomerase purified from brewers' yeast (Krietsch et al., 1970), as shown in Table II. The differences between the two sets of results can be attributed to different assay conditions. The results for the brewers' yeast enzyme were conducted at 25 °C rather than 30 °C, and under slightly different conditions of buffer concentration and ionic strength. The inhibition constants (K_i) of three substrate analogues (*RS*)-glycerol phosphate, 2-phosphoglycolate (Wolfenden, 1969), and phosphoglycolohydroxamate (Collins, 1974), were determined and are listed in Table II. The inhibition constant for arsenate, a necessary factor in the determination of K_m^+, is also given in Table II.

[1(*R*)-^{3}H]Dihydroxyacetone phosphate that had been prepared with chicken muscle triosephosphate isomerase, an enzyme of known stereospecificity, was allowed to react with the yeast isomerase. The specific radioactivity of the remaining

Table III: Incorporation of Solvent Tritium into Remaining Substrate Dihydroxyacetone Phosphate and into Product 3-Phosphoglycerate, in the Reaction of Dihydroxyacetone Phosphate Catalyzed by the Yeast Isomerase

fractional extent of reaction ($1 - r$)	specific radioactivity of solvent, x (μCi/μmol)	specific radioactivity of remaining substrate, s (μCi/μmol)	specific radioactivity of product, p (μCi/μmol)
0.50	0.168 ± 0.009	0.028 ± 0.002[a]	0.078 ± 0.002[b]
0.85	0.16 ± 0.01	0.145 ± 0.05[c]	0.083 ± 0.001[d]

[a] s/x is 0.17 ± 0.01. [b] p/x is 0.46 ± 0.03. The correction for incomplete exchange is 0.01, giving a value for A_8' of 0.47 [see Maister et al. (1976)]. [c] s/x is 0.9 ± 0.3. [d] p/x is 0.53 ± 0.04. The correction for incomplete exchange is 0.009, giving a value for A_8' of 0.54 [see Maister et al. (1976)].

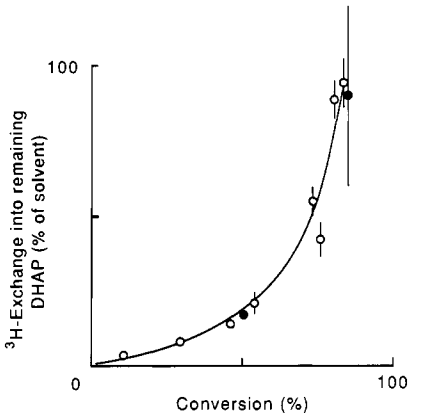

FIGURE 2: Exchange–conversion plot for the chicken (○) and yeast (●) triosephosphate isomerases with dihydroxyacetone phosphate as substrate. The data for the chicken isomerase are from Maister et al. (1976) and are fit to $A_6' = 0.50$ and eq 6.6 from Albery and Knowles (1976a).

substrate, s (expressed as a fraction of the specific radioactivity of the substrate at the start of the reaction, s_0), at 66% reaction was 1.95 ± 0.1. The amount of tritium transferred from the labeled substrate to the ultimate product 3-phosphoglycerate, p (expressed as a fraction of s_0), at 66% reaction was 0.032 ± 0.002. Isomerase reactions were routinely quenched by acidification to below pH 2.5 and cooling to 0 °C. Independent experiments demonstrated that the enzyme is catalytically inactive at pH values below 3. The absence of catalytic activity is essential for the validity of the ^{3}H transfer and ^{3}H exchange studies, and in all such experiments, the pH and temperature were kept low until all traces of the isomerase had been removed.

With dihydroxyacetone phosphate as substrate, a study of the effects of isotopic substition of deuterium at the 1-*pro-R* position was made. The value of k_{cat}^H/k_{cat}^D for dihydroxyacetone phosphate is 3.4 ± 0.1, while the K_m values are largely unaffected by isotopic substitution ($K_m^H/K_m^D \approx 1$), as shown by the nonlinear least-squares fitting program ISOVKVC (Cleland, 1979), which does not assume equal effects on V and on V/K. A much smaller deuterium isotope effect on k_{cat} was seen when the rate of reaction of [2-^{2}H]glyceraldehyde 3-phosphate with the yeast enzyme was compared to that of unlabeled glyceraldehyde 3-phosphate. The value of k_{cat}^H/k_{cat}^D obtained for glyceraldehyde phosphate is 1.6 ± 0.1.

The appearance of solvent tritium in the remaining substrate dihydroxyacetone phosphate and in the product glyceraldehyde 3-phosphate (which is converted in situ to 3-phosphoglycerate) was determined at two extents of reaction. Values for the specific radioactivity of remaining dihdyroxyacetone phosphate, s (expressed as a fraction of the specific radioactivity of the solvent, x), are shown in Table III and are plotted in Figure 2 as a function of the extent of reaction, $1 - r$, along with results obtained with the chicken muscle isomerase (Maister et al., 1976). The values for the specific radioactivity of the product 3-phosphoglycerate, p (expressed as a function of x), are also shown in Table III. These values represent the discrimination against the appearance of solvent tritium in the product. A similar experiment was performed in the reverse direction of reaction, with glyceraldehyde 3-phosphate as the substrate. The appearance of solvent tritium in the remaining substrate glyceraldehyde 3-phosphate and in the product dihydroxyacetone phosphate (which is converted in situ to glycerol phosphate) was determined at three extents of reaction. The values obtained for the specific radioactivity of substrate glyceraldehyde 3-phosphate, p (expressed as a fraction of x), are shown in Table IV and are plotted as a function of the extent of reaction in Figure 3 along with values from the analogous experiment of Fletcher et al. (1976) with the chicken isomerase. The values for the discrimination against tritium incorporation into the ultimate product glycerol phosphate, s (expressed as a fraction of x), are also listed in Table IV.

Table IV: Incorporation of Solvent Tritium into Remaining Substrate Glyceraldehyde 3-Phosphate and into Product Glycerol 3-Phosphate, in the Reaction of Glyceraldehyde 3-Phosphate Catalyzed by the Yeast Isomerase

fractional extent of reaction ($1 - r$)	specific radioactivity of solvent, x (μCi/μmol)	specific radioactivity of remaining substrate, p (μCi/μmol)	specific radioactivity of product, s (μCi/μmol)
0.28	0.161 ± 0.005	0.0074 ± 0.0005[a]	0.021 ± 0.0025[b]
0.61	0.182 ± 0.009	0.044 ± 0.003[c]	0.0181 ± 0.0007[d]
0.87	0.168 ± 0.005	0.068 ± 0.003[e]	0.0158 ± 0.0004[f]

[a] p/x is 0.46 ± 0.003. [b] s/x is 0.13 ± 0.02. The correction for incomplete exchange is 0.004, giving a value for B_8' of 0.134 [see Fletcher et al. (1976)]. [c] p/x is 0.24 ± 0.02. [d] s/x is 0.100 ± 0.007. The correction for incomplete exchange is 0.003, giving a value for B_8' of 0.103 [see Fletcher et al. (1976)]. [e] p/x is 0.41 ± 0.02. [f] s/x is 0.094 ± 0.002. The correction for incomplete exchange is 0.001, giving a value for B_8' of 0.095 [see Fletcher et al. (1976)].

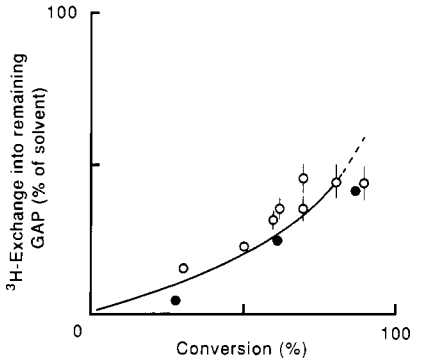

FIGURE 3: Exchange–conversion plot for the chicken (○) and yeast (●) triosephosphate isomerases with glyceraldehyde phosphate as substrate. The data for the chicken isomerase are from Fletcher et al. (1976) and are fit to $B_6' = 1.14$, $B_7' = 0.3$, and eq 1 from Fletcher et al. (1976).

Discussion

The determination of the free energy profile for triosephosphate isomerase requires the combination of kinetic data from a variety of different types of experiments using both tracer tritium labeling in each substrate and in the solvent and stoichiometric deuterium labeling in each of the two substrates. The experiments reported here on the yeast isomerase are compared to the results previously obtained with the chicken

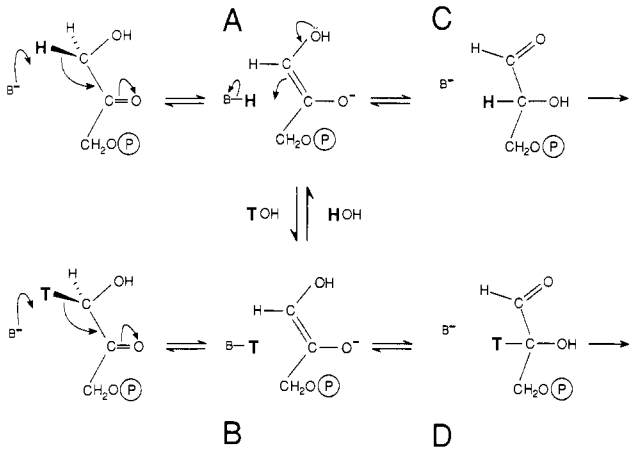

FIGURE 4: Mechanistic pathway for the triosephosphate isomerase catalyzed reaction. All species are enzyme bound.

enzyme and show that these two enzymes are energetically very similar. The derived free energy profile for the yeast isomerase further serves as a basis for comparison with mutant yeast isomerases, and one such comparison is presented in the following paper (Nickbarg et al., 1988).

First, the existence of a reaction intermediate in which a catalytic group on the enzyme exchanges its proton with the solvent is inferred from the result of the tritium transfer experiment, in which stereospecifically labeled [1(*R*)-^{3}H]dihydroxyacetone phosphate is allowed to react with the isomerase under irreversible conditions (that is, under conditions where the product of the isomerase reaction is rapidly removed by a coupling enzyme system). For the chicken isomerase, very little of the substrate tritium (3–6%, depending on the extent of reaction) is found in the isolated product (Herlihy et al., 1976). The native yeast isomerase shows a comparable loss of label under similar conditions. Since in the absence of isomerase neither dihydroxyacetone phosphate nor glyceraldehyde phosphate exchanges protons with the solvent to a significant degree, the enzymatic reaction must proceed via a reaction intermediate that is in rapid protonic exchange with the medium. The obvious candidate for such a species is the enzyme-bound *cis*-enediol or enediolate intermediate (Rieder & Rose, 1962). In terms of the mechanism illustrated in Figure 4, A and B are in rapid equilibrium. The amount of tritium left in the remaining substrate during such a transfer experiment is determined by two opposing factors. The first factor, the primary tritium isotope effect in the proton abstraction step (i.e., the discrimination against tritiated molecules in the conversion of bound substrate to intermediate, see Figure 4), results in the accumulation of tritium in the remaining substrate which causes the specific radioactivity of the remaining pool of substrate molecules to rise over the course of the reaction. The second factor is the washout of the substrate label into the solvent medium via the enzyme-bound enediol intermediate (B to A, see Figure 4), a process that lowers the specific radioactivity of the remaining substrate pool. The importance of this second factor depends upon the partition ratio of the enzyme-bound enediol intermediate, that is, the rate at which the intermediate reacts forward to yield product versus the rate at which it collapses back to re-form the substrate. A partition ratio favoring substrate allows the substrate pool to preequilibrate with the solvent and to wash out the substrate's isotopic label faster than substrate is converted to product. Analysis of the remaining [1(*R*)-^{3}H]dihydroxyacetone phosphate after partial reaction with the yeast isomerase shows that the remaining substrate is *enriched* in tritium compared to the substrate at the start of the reaction. Since the specific radioactivity of the remaining substrate is a function of the extent to which the proton transfer step is rate limiting in the formation of the enediol intermediate, the manifestation of the primary tritium isotope effect means that the rate of formation of the intermediate is dominated by the rate of abstraction of the 1-*pro-R* proton from dihydroxyacetone phosphate.

The existence of a enzyme-bound intermediate in rapid protonic exchange with the solvent (i.e., A $\rightleftharpoons$ B, see Figure 4) makes it possible to feed ^{3}H from tritiated water both into the remaining substrate and into the product. First, the discrimination between hydrogen isotopes in the collapse of the intermediate to give product can be observed. Since A and B (Figure 4) are at equilibrium, the relative rate of A (via C) to give unlabeled product and of B (via D) to give labeled product is measured in this experiment. Second, since only the intermediate exchanges with solvent and this exchange occurs rapidly, the partitioning of the intermediate between breakdown back to substrate and reaction forward to give product can also be observed. If the substrate equilibrates its proton with the solvent (via the intermediate A $\rightleftharpoons$ B) faster than the intermediate is converted irreversibly to product, then the remaining reactant will quickly reach isotopic equilibrium with the solvent. On the other hand, if the intermediate collapses to form product much faster than it partitions back to the substrate in what Northrop (1977) has called a high forward commitment, very little solvent isotope will be incorporated into the remaining substrate. In the present case, the reaction can be studied in each reaction direction, and the two extreme cases outlined above are complementary. For instance, if the intermediate partitions more readily toward product in the forward reaction direction (and therefore shows little solvent isotope in the remaining substrate pool), the same intermediate will, when formed in the reverse reaction direction, allow the preequilibration between the solvent isotope and the starting material.

When dihydroxyacetone phosphate is the substrate (Figure 4, from left to right), the specific radioactivity of the product glyceraldehyde 3-phosphate is not very far from that of the solvent in which the reaction is run. With the chicken isomerase, the discrimination against tritium is only 1.3-fold (Leadlay et al., 1976), and the corresponding value for the yeast isomerase reaction is 2-fold. These numbers are much smaller than expected values of primary tritium kinetic isotope effects (of 6–20-fold; Bell, 1973), which suggests that the proton transfer step in which the intermediate collapses to form glyceraldehyde phosphate is not cleanly rate limiting for this half of the reaction. Since isotopic discrimination only illuminates the reaction steps that follow the solvent exchange event, we must conclude that the transition state for a step *following* the protonation at C-2 (i.e., after the formation of C and D, Figure 4) is rate limiting. The most likely candidate for such a rate-determining transition state is that which involves the release of the product glyceraldehyde phosphate from the enzyme. In contrast to this behavior, when the reaction is run in the reverse direction with glyceraldehyde phosphate as substrate (Figure 4, from right to left), the specific radioactivity of the product dihydroxyacetone phosphate (ultimately glycerol phosphate) is only 11% that of solvent. This corresponds to a 9-fold discrimination against tritium, a value that is in the range expected for a primary tritium isotope effect. With glyceraldehyde phosphate as substrate, therefore, the product-forming transition state is that in which proton transfer occurs. While the discrimination

against solvent tritium in the formation of product provides information about the relative rates of the product-forming steps that follow the enediol intermediate, these data do not shed light upon the partition ratio of the intermediate itself. To discern how the intermediate partitions forward to product or back to substrate, we must investigate the incorporation of solvent isotope into the remaining substrate.

With dihydroxyacetone phosphate as substrate in tritiated water, the appearance of tritium in the remaining substrate follows the same trends in both the chicken and yeast isomerase catalyzed reactions. The plot of the specific radioactivity of tritium in remaining dihydroxyacetone phosphate versus the extent of the reaction does not correspond to either of the extreme cases outlined earlier, that is, either complete equilibration of remaining substrate with solvent protons early on in the reaction or no incorporation of solvent isotope into remaining substrate even at late reaction times. Rather, we see for both enzymes a situation where the partitioning of the enediol intermediate does not particularly favor the formation of either the product or the starting material. As is evident from Figure 2, the specific radioactivity of remaining dihydroxyacetone phosphate rises slowly (with a slope of about 0.3 at early extents of reaction), then curves upward in the later stages of the reaction. The initial slope of this plot indicates that the enediol intermediate reacts forward to give free glyceraldehyde phosphate (that is "conversion") about 3 times faster than it falls back having exchanged with solvent ^3H to yield labeled dihydroxyacetone phosphate (this is "exchange"). As the reaction proceeds, the substrate pool (which started unlabeled) contains increasing amounts of 1(*R*)-^3H-labeled molecules. Now the existence of a primary kinetic tritium isotope effect in the proton abstraction step for these species means that unlabeled substrate will react *out* of the pool faster than tritiated material. As the reaction proceeds, therefore, the shrinking substrate pool becomes increasingly enriched in 1(*R*)-^3H-labeled species, resulting in the pronounced upward curvature of the exchange–conversion plot seen in Figure 2.

As a check on the validity of the conclusions from Figure 2, an exchange–conversion experiment can be run in the reverse direction with glyceraldehyde phosphate as substrate. This exchange–conversion plot follows a similar but not identical pattern. The initial gradient of the specific radioactivity plot of remaining glyceraldehyde phosphate versus the extent of reaction shows that the enzyme-bound intermediate partitions forward to dihydroxyacetone phosphate about 3 times faster than it collapses back to yield 2-^3H-labeled glyceraldehyde phosphate.

The use of deuterium-labeled substrates can provide results that are complementary to those obtained through the use of tritium. Since deuterium labeling is normally stoichiometric, the use of ^2H-labeled substrates allows direct evaluation of the kinetic importance of particular proton transfer steps in the overall reaction and thus links the steady-state kinetic parameters determined with unlabeled material and the partition ratios determined from tritium discrimination and exchange–conversion experiments. For instance, the low value for the discrimination against tritium in the formation of glyceraldehyde phosphate shows that enzyme-bound glyceraldehyde phosphate and the enediol intermediate are largely in equilibrium with each other and with solvent (in terms of Figure 4, A, B, C, and D are largely at equilibrium). Therefore, when [2-^2H]glyceraldehyde phosphate is used as substrate, a low deuterium isotope effect is expected because the isotopic label will be mostly washed out to solvent before the rate-limiting conversion to product. This is precisely what is observed. Chicken isomerase yields a deuterium isotope effect in k_{cat} of 1.1 ± 0.1 for deuteriated glyceraldehyde phosphate (Leadlay et al., 1976). That for the yeast isomerase is slightly larger, at 1.6 ± 0.1. The higher value observed with the yeast isomerase is consistent with a slightly larger tritium discrimination value (of 2.0) compared with that observed with the chicken isomerase (of 1.3).

Table V: Values of A_n and B_n Parameters for the Yeast Triosephosphate Isomerase Expressed in *E. coli* DF502(p12)

$A_1 = (1.9 \pm 0.1) \times 10^{-6}$ M s	$B_1 = (6.3 \pm 0.5) \times 10^{-9}$ M s
$A_2 = (1.33 \pm 0.04) \times 10^{-3}$ s	$B_2 = (1.14 \pm 0.04) \times 10^{-4}$ s
$A_3/A_1 = 3.1 \pm 0.3$	$B_3/B_1 = 1.3 \pm 0.1$
$A_4/A_2 = 3.7 \pm 0.4$	$B_4/B_2 = 1.8 \pm 0.1$
$A_5' = 0.063 \pm 0.005$	$B_5' = 0.023 \pm 0.006$
$A_6' = 0.38 \pm 0.04$	$B_6' = 1.09 \pm 0.02$
$A_7' = 0.27 \pm 0.03$	$B_7' = 0.21 \pm 0.06$
$A_8' = 0.71 \pm 0.01$	$B_8' = 0.11 \pm 0.02$
$\Phi_S = 1.03 \pm 0.04$[a]	$\Phi_P = 1.0 \pm 0.2$[a]

[a] Data from Fletcher et al. (1976).

When dihydroxyacetone phosphate is used as substrate, we know from the tritium experiments described above that the rate-limiting transition state is that involving the release of product glyceraldehyde phosphate from the enzyme. What do we then predict for the behavior of [1(*R*)-^2H]dihydroxyacetone phosphate? The fast solvent exchange that occurs at the enediol intermediate means that once the 1(*R*)-^2H label has been abstracted by the enzyme it will be irretrievably lost to solvent. Since, after this label loss, the reactions of initially labeled and initially unlabeled substrate out of the enediol pool will be identical, any differences in reaction rate of the ^2H-labeled species will be determined by the flux of molecules entering the enediol pool. A full primary deuterium isotope effect is expected when labeled dihydroxyacetone phosphate is the substrate, even though the proton abstraction step is not rate limiting for the overall reaction. The deuterium isotope effect in k_{cat} for dihydroxyacetone phosphate and the yeast isomerase is 3.4 ± 0.1, while the analogous value with the chicken isomerase is 2.9 ± 0.1 (Leadlay et al., 1976).

Thus far, we have discussed the yeast isomerase free energy profile only in qualitative terms. Calculation of the profile requires the synthesis of the results from all the various classes of experiments, following the route described earlier (Albery & Knowles, 1976a) for chicken muscle triosephosphate isomerase. Each experiment allows the evaluation of one or more parameters, A_n and B_n, which comprise various rate constants and isotopic fractionation factors. The A_n parameters relate to experiments run in the S to P direction (where S is dihydroxyacetone phosphate and P is glyceraldehyde phosphate), and the B_n parameters relate to experiments run in the P to S direction. The individual rate constants and fractionation factors are then derived from these parameters according to the methods outlined earlier (Albery & Knowles, 1976b).

Several of the A_n parameters (and their B_n counterparts) can be derived from more than one experiment. This allows several internal checks on the data used to construct the energy profile. While the data for the yeast enzyme are less extensive than those used originally to determine the energetics of the chicken muscle isomerase, the behavior of the yeast mutant can be unambiguously defined. The A_n and B_n parameters, calculated according to the procedures described in Albery and Knowles (1976a) and Albery and Knowles (1976b), are listed in Table V. From these values, the parameter θ, which describes the overall partitioning of the enzyme-bound enediol between S and P in the all-hydrogen system, can be derived

Table VI: Comparison of Kinetic Parameters for the Yeast and Chicken Triosephosphate Isomerases[a,b]

parameter	yeast enzyme	chicken enzyme[c]
$\Phi_{1,2}$	0.11 ± 0.04	0.14 ± 0.01
$\Phi_{3,4}$	0.54 ± 0.07	0.83 ± 0.01
θ	1.7 ± 0.3[d]	2.2 ± 0.3[d]
θ	4.1 ± 1.8[e]	2.1 ± 0.3[e]
θ	2.9 ± 1.7[f]	2.2 ± 0.2[f]
k_1/k_{-1} (M^{-1})	600 ± 300[g]	800 ± 200[g]
k_2/k_{-2}	0.1 ± 0.5[h]	0.3 ± 0.3[h]
k_3/k_{-3}	$[(1.7 \pm 0.8) \times 10^{-4}]/[(1 \pm 6) \times 10^{-4}]$[i]	$(0.0 \pm 0.4)/(0.6 \pm 0.6)$[i]
k_4/k_{-4} (M)	$(4 \pm 2) \times 10^{-5}$[j]	$\geq 3 \times 10^{-4}$[j]

[a] Calculated from values listed in Table V and Albery and Knowles (1976a). [b] The values are corrected for the equilibrium proportion of hydrated substrate forms. [c] Data from Albery and Knowles (1976b). [d] From A_n' data. [e] From B_n' data. [f] Mean value. [g] Using eq 9.7 of Albery and Knowles (1976a). [h] Using eq 9.8 of Albery and Knowles (1976a). [i] Using eq 9.9 of Albery and Knowles (1976a). [j] Using eq 9.10 of Albery and Knowles (1976a).

from either the A_n or the B_n results. The values for θ are shown in Table VI and indicate that the enzyme-bound enediol intermediate favors the formation of S about 3 times more than the formation of P. This preference is very close to that shown by the chicken isomerase (Albery & Knowles, 1976b). Also shown in Table VI are values for the fractionation factors $\Phi_{1,2}$ and $\Phi_{3,4}$. The low value of $\Phi_{1,2}$ (0.11) shows that the proton transfer step (the conversion of enzyme-bound dihydroxyacetone phosphate to enzyme-bound enediol) dominates and that the binding of dihydroxyacetone phosphate is relatively fast. In contrast, the relatively high value of $\Phi_{3,4}$ (0.54) indicates that the protonation of the enediol intermediate to enzyme-bound glyceraldehyde phosphate is as fast or faster than the release of glyceraldehyde phosphate from the enzyme. If the transition state for the glyceraldehyde phosphate release step were *completely* rate limiting, we should expect $\Phi_{3,4}$ to be equal to unity. That it is not shows that the transition state for the proton transfer step still contributes somewhat to the overall formation of free glyceraldehyde phosphate from the enzyme-bound enediol. The partition ratios of the three intermediates ($\alpha = k_2/k_{-1}$, $\beta = k_{-2}/k_3$, and $\gamma = k_{-3}/k_4$) are also derived from the A_n and B_n data, and from these results, the thermodynamic data in Table VI can be obtained. These values have been calculated for the unhydrated forms of the substrates (which are those actually bound and used by the isomerase) and are compared to the analogous values derived for the chicken isomerase in Table VI.

To illustrate the kinetic results in the form of a free energy profile, we need to define a standard state (so that the second-order rate constants relating to substrate binding can be presented along with the first-order rate constants relating all enzyme-bound forms). In our earlier work on the chicken muscle enzyme, a standard state of 40 μM was chosen, since this is the (relatively invariant) concentration of triose phosphate in muscle (Williamson, 1965). The concentration of metabolites in yeast cells is rather more variable and is dependent on changes in nutrient conditions (Eddy, 1958) and upon the extraction procedures used in the determination (Gancedo & Gancedo, 1973). An average dihydroxyacetone phosphate concentration of 3 mM has been found in *Saccharomyces carlsbergensis* (Hess et al., 1969), and values for triose phosphate concentrations in yeast extracts ranging from 50 to 170 μM have been reported (Gancedo & Gancedo, 1973). In the absence of definite values for the intracellular triose phosphate concentration in yeasts, we have chosen 40 μM in order to allow direct comparison of the present results with those previously obtained with the isomerase from chicken muscle. The derived free energy profile of the yeast isomerase is shown in Figure 5.

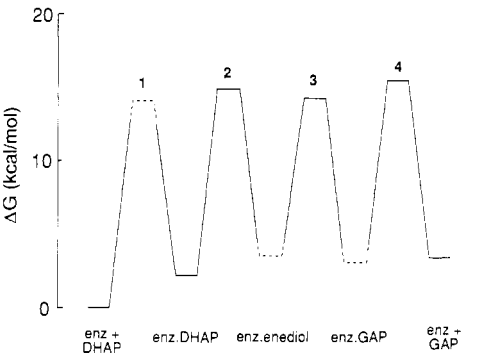

FIGURE 5: Free energy profile for the reaction catalyzed by yeast triosephosphate isomerase. The dashed parts of the profile are less precisely established, and the rate constants involving these states are lower limits.

The free energy profile shows that the enzyme-bound complexes are kinetically insignificant and that the rates of the enzyme-catalyzed proton transfer steps are not rate limiting for the overall second-order reaction. This illustrates the reason for the extraordinary efficiency of the yeast enzyme. The highest transition state for the catalyzed reaction is that for the binding of the less stable substrate, glyceraldehyde phosphate, to the enzyme, and the rate constant for this reaction (2×10^8 M^{-1} s^{-1}) suggests that this is a diffusive process. Recent results from viscosity variation experiments on the chicken muscle enzyme (Blacklow et al., 1988) confirm this view. The most stable state (at the in vivo substrate levels experienced by the enzyme) is that of the enzyme plus free dihydroxyacetone phosphate. These two facts led us earlier to conclude that (at these substrate concentrations and assuming freely diffusible species) the chicken muscle triosephosphate isomerase could not improve as a catalyst (Albery & Knowles, 1976c, 1977; Knowles & Albery, 1977). It appears from Figure 5 that the yeast enzyme may have attained a similar state of kinetic refinement.

The resemblance between the free energy profile for the yeast isomerase and that from chicken muscle isomerase is close, in spite of the considerable distance that separates yeasts from vertebrates on the phylogenetic tree. However, similarities between the intron positions in the gene sequences of the maize and chicken isomerases suggest that the isomerase gene predates the plant–animal divergence (Marchionni & Gilbert, 1986) and support the hypothesis that the catalytic power of triosephosphate isomerase developed early in evolution. Although the yeast and chicken isomerases show differences of 50% in their amino acid sequences, and have very different isoelectric points, the kinetic properties of these two catalysts as illustrated by their free energy profiles are virtually identical.

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