Secondary H/T and D/T Isotope Effects in Enzymatic Enolization Reactions. Coupled Motion and Tunneling in the Triosephosphate Isomerase Reaction†

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ABSTRACT: Secondary k_H/k_T kinetic isotope effects in H_2O and k_H/k_T or k_D/k_T isotope effects in D_2O have been measured for the triosephosphate isomerase-catalyzed conversion of dihydroxyacetone 3-phosphate (DHAP) to D-glyceraldehyde 3-phosphate. The proton transfer steps are made rate-limiting using [1(*R*)-^{2}H]-labeled substrate in D_2O to slow the chemical steps, relative to product release. After a small correction for the β-equilibrium isotope effect for dehydration of DHAP, the H/T kinetic isotope effect $k_H/k_T = 1.27 \pm 0.03$ for [1(*R*)-^{2}H,(*S*)-^{3}H]-labeled substrate in D_2O is subtantially larger than the equilibrium isotope effect for enolization of DHAP, $K_H/K_T = 1.12$. The H/T isotope effect is related to the D/T isotope effect with a Swain−Schaad exponent $y = 4.4 \pm 1.3$. These results are consistent with coupled motion of the C-1 primary and secondary hydrogens of DHAP and tunneling. Large secondary kinetic isotope effects are a general feature of enzymatic enolization reactions while nonenzymatic enolization reactions show secondary kinetic isotope effects that are substantially smaller than equilibrium effects [Alston, W. A., II, Haley, K., Kanski, R., Murray, C. J., & Pranata, J. (1996) *J. Am. Chem Soc., 118,* 6562−6569]. Possible origins for these differences in transition state structure are discussed.

The mechanisms by which enzymes activate the α-proton of a carbon adjacent to a carbonyl group are of intense theoretical and experimental interest (Thibblin & Jencks, 1979; Knowles, 1991; Gerlt & Gassman, 1992, 1993a,b). Estimates of the stabilities of enzyme-bound enolate intermediates suggest that enzymes stabilize these intermediates or avoid their formation through concerted reaction mechanisms (Jencks, 1981; Amyes & Richard, 1992; Cleland & Kreevoy, 1994).

In view of the simplicity of the proton transfers catalyzed by triosephosphate isomerase, this enzyme serves as a good model for examining how enzymes handle moderately unstable carbanion intermediates. Extensive kinetic (Rose, 1958; Rieder & Rose, 1959; Webb & Knowles, 1972; Knowles, 1991), structural (Belasco & Knowles, 1980; Alber et al., 1981; Lolis et al., 1990; Davenport et al., 1991; Komives et al., 1991; Lodi & Knowles, 1991, 1993; Zhang et al., 1994), and theoretical (Albery, 1982; Bash et al., 1991; Alagona et al., 1995) studies have established that (1) glutamate-165 abstracts the primary (*pro-R)* proton from DHAP and (2) histidine-95 acts as an electrophilic catalyst to polarize the carbonyl group in the enzyme−substrate complex in the initial step of the enzyme-catalyzed conversion of DHAP to GAP as shown in Figure 1.

For example, the 19 cm^{-1} reduction in the C=O stretching frequency of DHAP when it is bound to the enzyme is consistent with electrophilic activation of the substrate (Belasco & Knowles, 1980). It has been suggested that this polarization proceeds with proton transfer to oxygen to form the enediol−imidazolate ion intermediate in Figure 1 with a late transition state (Gerlt & Gassman, 1993b). However,

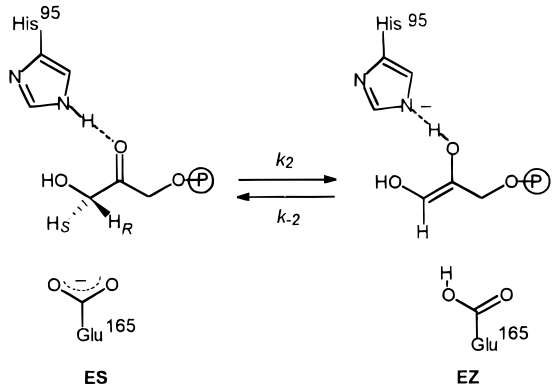

FIGURE 1: Proposed reaction pathway for TIM-catalyzed conversion of DHAP to a enediol intermediate by the active-site base, glutamate-165, and histidine-95, as an electrophilic catalyst. The primary *pro-R* proton is transferred and the secondary *pro-S* proton remains bound to the C-1 carbon of the enediol. The terms ES and EZ and the rate constants k_2 and k_{-2} refer to the species and rate constants in Scheme 1.

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[1] Abbreviations: CHAP, 1-chloro-3-hydroxyacetone phosphate; DHAP, dihydroxyacetone 3-phosphate; EDTA, ethylenediaminetetraacetate; GAP, (*R*)-glyceraldehyde 3-phosphate; GAPDH, rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase; HPLC, high pressure liquid chromatography; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; [^{32}P]NAD^+, nicotinamide adenine dinucleotide-*adenylate*-^{32}*P*; NMR, nuclear magnetic resonance; TEA HCl, triethanolamine hydrochloride; TIM, yeast triosephosphate isomerase. The term "hydron" refers to the hydrogen cation (L^+) without regard to nuclear mass.

[2] A neutral imidazole has a pK_a of 14.5. The electrophilicity of the neutral imidazole may be enhanced by the field induced by the helix dipole formed by residues 95−102 (Lodi & Knowles, 1993).

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it is difficult to establish the nature and degree of proton transfer in the transition state from structural studies and kinetic evidence for the formation of an enediol-like transition state is indirect (Raines et al., 1986; Nickbarg et al., 1988). Similar ambiguities exist in the mechanism of action of other enzymes that generate enol(ate) intermediates (Gerlt & Gassman, 1992, 1993a,b; Hawkinson & Pollack, 1993; Stivers et al., 1996).

Because an understanding of enzyme catalysis fundamentally requires an understanding of transition-state structure (Schowen, 1978), we sought to establish the nature and degree of bond-breaking in enzymatic enolization reactions using secondary k_H/k_T and k_D/k_T isotope effects. Secondary kinetic isotope effects are sensitive to the vibrational frequencies of the C−H bond and are expected to vary between unity and the corresponding equlibrium isotope effect (Cleland, 1987). In the triosephosphate isomerase reaction, cleavage of the primary 1-*pro-R* proton of DHAP by Glu-165 results in a change from sp^3 to sp^2 hybridization of the secondary *pro-S* C−H bond (Figure 1) that gives rise to a large secondary isotope effect $k_H/k_T = 1.27 \pm 0.03$. With an understanding of α-secondary isotope effects on simple enolization reactions in hand (Alston et al., 1996), we show here that large H/T and D/T secondary kinetic isotope effects in C−H bond cleavage reactions are a general feature of enzyme reactions that involve enol(ate) intermediates and these large effects provide evidence for a change in the *nature* of the transition state for enolizaton reactions relative to non-enzyme-catalyzed enolizations.

MATERIALS AND METHODS

Materials. Yeast TIM, GAPDH, rabbit muscle aldolase, fructose 1,6-bisphosphate trisodium salt, NAD^+, and NADH were from Boehringer Mannheim Ltd. (Indianapolis, IN). Enzymes were desalted, and any contaminating TIM was removed from aldolase and GAPDH by treatment with 1-chloro-3-hydroxyacetone phosphate followed by ultrafiltration through Centricon-10 concentrators (10K MW cutoff) from Amicon, Inc. (Beverly, MA) in a procedure similar to that previously described (Nickbarg & Knowles, 1988). Dicyclohexylammonium 2,2-dimethoxy-1,3-propanediol phosphate, dicyclohexylammonium 3-phosphoglycerate, and TEA HCl were from Sigma Chemical Co. (St. Louis, MO). DHAP was prepared by acid hydrolysis of the ketal on Dowex (50 × 4−200-R) cation exchange resin. Deuterium oxide (D_2O, 99.9 atom % D) and deuterium chloride (35% DCl; 99.9 atom % D) were from Isotec, Inc. (Miamisburg, OH). Tritiated water (TOH; 5 Ci /mL) and [^{32}P]NAD^+ (250 Ci/mmol) were from ICN Biomedicals, Inc. (Costa Mesa, CA). Tritiated water was diluted to a specific activity of 0.1 Ci/mL in H_2O or D_2O. [^{32}P]NAD^+ was diluted to a specific activity of 0.11−0.33 Ci/mol and used within 36 h of the radiochemical synthesis by the manufacturer. 1-Chloro-3-hydroxyacetone phosphate (CHAP) was synthesized as previously described (Silverman et al., 1975). All other reagents were from commercial sources and were used without further purification.

Methods. Radioactivity was measured with a Wallac-1410 liquid scintillation counter using 1 mL aqueous samples in 5 mL of Optiphase Hisafe 3 scintillation cocktail from Wallac-Oy (Turku, Finland). pH determinations were made on an Accumet 910 pH meter (Fisher Scientific) with a Radiometer GK2321C combination electrode. In D_2O solutions the pD was obtained by adding 0.4 to the observed pH (Glasoe & Long, 1960). Ultraviolet−visible spectroscopy was performed on a Hitachi U-2000 spectrophotometer. Proton NMR spectra were recorded on JEOL FX90Q or Varian VXR-500S instruments. HPLC separations were performed with a Shimadzu LC-6A HPLC system with an SPD-M6A diode array detector.

[1(S)-^{3}H]Dihydroxyacetone Phosphate (**1**). DHAP (69 μmol; 0.7 mL) was added to 0.15 M TEA buffer, pH 7.4 (0.4 mL), in the presence of aldolase (40 units). Tritiated water (TOH; 0.1 Ci/mL; 0.5 mL) was added to give a final specific radioactivity of 0.56 Ci/mol. The mixture was allowed to stand at room temperature for 2 h and then freeze-dried to remove the bulk of the tritiated water. The residue was dissolved in 2 mL of 0.16 mM HCl, pH 3.8, and the enzyme was removed by ultrafiltration in a Centricon-10 concentrator at 5000*g* for 30 min. The filtrate was loaded on a Dowex-1 (Cl^-) anion exchange column (6.5 cm × 0.56 cm^2) that was equilibrated with 0.16 mM HCl. The column was washed with 0.16 mM HCl until the radioactivity of the effluent was close to background (∼300 dpm/50 μL). The column eluent was switched to 100 mM HCl, and [1(*S*)-^{3}H]DHAP was collected in 2 mL fractions. Fractions containing [1(*S*)-^{3}H]DHAP were concentrated by bulb-to-bulb distillation. The pH was adjusted to pH 4, and the samples with a specific radioactivity of 0.23 Ci/mol were stored in 50 μL aliquots at −20 °C. Greater than 99% of the tritium was incorporated in the *pro-S* position of [1-^{3}H]-DHAP as determined by incubating [1(*S*)-^{3}H]DHAP in the presence of (a) 300 units of aldolase, (b) 8500 units of TIM, and in 0.05 M TEA HCl, pH 7.4, for 1 h followed by bulb-to-bulb distillation.

[1(R,S)-^{3}H]Dihydroxyacetone Phosphate (**2**). Nonstereospecifically tritium labeled DHAP was prepared in H_2O as follows. The reaction mixture contained 110 mM TEA HCl buffer, pH 7.6, 22 μmol of DHAP, tritiated water (0.2 Ci; final specific activity of 0.73 Ci/mol), and TIM (8500 units) in 4.9 mL. The mixture was left at room temperature for 2 h. Triosephosphate isomerase was removed by ultrafiltration at 3000*g* for 40 min at 5 °C on a Centricon-10 concentrator. Isomerase free aldolase (40 units) was then added to convert the equilibrated mixture of triosephosphates into fructose 1,6-bisphosphate and DHAP as well as to exchange the remaining 1-*pro-S* proton of DHAP. After incubation at room temperature for 2 h, aldolase was removed on a Centricon-10 concentrator and the reaction mixture was quenched by acidification to pH 4.0. The solvent was removed by bulb-to-bulb distillation over 5 h and the residue applied to a Dowex-1-Cl^- column (9 cm × 1 cm^2) equilibrated with 0.16 mM HCl. The column was washed with 140 mL of 0.16 mM HCl. DHAP and fructose-1,6-bisphosphate were separated by a linear gradient from 0.16 to 100 mM HCl in 125 mL. Fractions (6 mL) were assayed for radioactivity and fractions containing DHAP (89% total counts) were pooled and concentrated by bulb-to-bulb distillation to give 18 μmol of [1(*R,S*)-^{3}H]DHAP in 2.5 mL with a specific activity of 0.73 Ci/mol. Enzymatic washout of tritium by aldolase or TIM showed that 49 ± 1% of the tritium was in the *pro-S* position and 51 ± 1% was in the *pro-R* position. HPLC analysis showed that >95% of the radioactivity comigrated with DHAP and 5% was tritiated water.

[1(R,S)-^{1}H]Dihydroxyacetone Phosphate in D_2O. Dowex (50 × 4−200-R) cation exchange resin, 2 g, was washed thoroughly with D_2O followed by vacuum filtration to remove all traces of water from the resin. The resin was added to a 4 mL of D_2O containing 50 mg (116 mmol) of dicyclohexylammonium 2,2-dimethoxy-1,3-propanediol phosphate, swirled for 30 s, and the resin removed rapidly by vacuum filtration. The filtrate was incubated at 40 °C for 8 h under N_2 to generate nondeuterated DHAP in D_2O (86 μmol). The pD of the solution was adjusted to 7.5 by the addition of anhydrous K_2CO_3. The extent of hydration of DHAP in D_2O was determined using 500 MHz ^{1}H NMR spectroscopy to integrate the areas of the C-3 protons for the hydrate (δ = 3.80 ppm) and ketone (δ = 4.46 ppm).

[1(R,S)-^{3}H,^{2}H]Dihydroxyacetone Phosphate (**3**). Nonstereospecifically labeled DHAP was prepared from DHAP in D_2O in a manner similar to [1(*R,S*)-^{3}H]DHAP. The reaction mixture contained (in 5.0 mL of D_2O) 200 mM TEA DCl buffer, pD 7.5, 60 μmol of [1(*R,S*)-^{1}H]DHAP in D_2O, TOD (0.2 Ci; final specific activity of 0.79 Ci/mol), and TIM (8500 units). After removal of TIM by ultrafiltration, isomerase free aldolase (40 units) was added. After the exchange was complete and aldolase was removed by ultrafiltration, the residue was applied to a Dowex-1-Cl$^-$ column (9 cm × 1 cm^2) equilibrated with 0.16 mM DCl. The column was washed with 140 mL of 0.16 mM DCl. DHAP and fructose 1,6- bisphosphate were separated by a linear gradient from 0.16 to 100 mM DCl in 125 mL. The final pooled material gave 53 μmol of [1(*R,S*)-^{3}H,^{2}H]DHAP in 2.8 mL with a specific activity of 0.74 Ci/mol. HPLC analysis showed that >95% of the radioactivity comigrated with DHAP and <5% was tritiated water. Rabbit muscle aldolase is known to exchange the C(3)-H of DHAP at a rate 400 times slower than the C(1)-H, so it is probable that **3** contains some deuterium at the C(3) position (Glades & Hill, 1978).

[1(R)-^{2}H]Dihydroxyacetone Phosphate. This compound was prepared as described by Leadlay et al. (1976). To the solution of nondeuterated DHAP in D_2O were added TIM (20 μL of a 10 mg/mL enzyme solution in D_2O) and 250 μL of 0.5 M TEA DCl, pD 7.5. The mixture stood at 20 °C for 1.5 h under N_2, and the reaction was quenched by placing the solution on ice and adding several milliliters of 1 M DCl to pD = 3.5. TIM was removed by ultrafiltration and the solution was stored at −40 °C. The equilibrated mixture of triosephosphates contained [1(*R*)-^{2}H]DHAP (80 μmol; 70% yield) with <5% nondeuterated DHAP by 500 MHz ^{1}H NMR spectroscopy.

[1(R)-^{2}H,1(S)-^{3}H]Dihydroxyacetone Phosphate (**4**). [1(*R*)-^{2}H]DHAP (60 μmol in D_2O; pD 3.6) containing 5% GAP was freeze-dried by bulb-to-bulb distillation and the residue dissolved in 100 mL of 0.16 mM HCl followed by bulb-to-bulb distillation. The residue was dissolved in 2 mL of 50 mM TEA buffer, pH 7.4, followed by the addition of isomerase free aldolase (30 units). Tritiated water (0.1 Ci/mL; 0.5 mL) was added to the reaction mixture to give a final specific radioactivity of 0.33 Ci/mol. The mixture was left at room temperature for 1.5 h, and the bulk of the tritiated water was removed by bulb-to-bulb distillation. The residue was dissolved in 0.16 mM HCl, and aldolase was removed by ultrafiltration through a Centricon-10 concentrator (5000*g* for 1 h at 5 °C). The filtrate was loaded on a Dowex-1 (Cl$^-$) anion exchange column (6.5 cm × 0.56 cm^2) equilibrated with 0.16 mM HCl. The column was washed with 0.16 mM HCl until the radioactivity of the wash was close to background (~100 dpm/50 μL). Tritiated DHAP and fructose 1,6-bisphosphate were eluted with a gradient from 0.16 to 50 mM HCl over 100 mL. Fractions (2 mL) were assayed for radioactivity, and fractions containing tritiated DHAP were pooled. The pH was adjusted to 4.0 with saturated potassium bicarbonate solution before storage at −40 °C overnight. The [1(*R*)-^{2}H,1(*S*)-^{3}H]DHAP solution was freeze-dried by bulb-to-bulb distillation, and the residue was dissolved in 0.5 mL of 0.16 mM DCl to give a 10.6 mM solution with a specific activity of 0.15 Ci/mol.

Secondary Kinetic Isotope Effects. Single-Label Experiments. The TIM-catalyzed production of GAP from DHAP was enzymatically coupled with oxidation of GAP by GAPDH/NAD$^+$ to give 3-phosphoglycerate and NADH with transfer of the *pro-S* (secondary) hydrogen to C-4 of NAD$^+$ (Rose, 1958; Reider & Rose, 1959). The concentration of DHAP was determined using a glycerol phosphate dehydrogenase/NADH assay.

Kinetic isotope effects were measured at pL 7.5 (L = H or D) in 50 mM TEA buffer at 30 °C. Typically, the reaction solution (2 mL) consisted of [1(*S*)-^{3}H]DHAP (0.6−1.2 mM), NAD$^+$ (1.5 mM), sodium arsenate (6 mM), EDTA (5 mM), and GAPDH (160 units). Control experiments showed that the TIM reaction was rate-limiting at [NAD$^+$] >0.3 mM. A reference sample (150 μL) was removed and quenched with 10 μL of a 3 mM solution of 1-chloro-3-hydroxyacetone phosphate (CHAP). The solution was equilibrated at 30 °C for 15 min, and the reaction was initiated by the addition of 20 μL of TIM (0.01 mg/mL enzyme in 20 mM TEA buffer; pH 7.5; 2.5 units). A portion of the solution (0.45 mL) was transferred to a 1 cm cuvette where the absorbance change at 375 nm was monitored (ϵ_{NADH} = 1625 M^{-1} cm^{-1}). At time intervals, 150 μL aliquots were transferred to Centricon-10 concentrators containing 10 μL of ~1 mM CHAP to quench the reaction. Control experiments showed that TIM was completely inactivated under these conditions. A "t_0" control experiment ± CHAP-inactivated TIM showed no production of [^{32}P]NADH that would be expected if hydrolysis of the active site ester formed in the quenching reaction was reversible under these conditions (de le Mare et al., 1972). At the completion of the reaction, a sample was transferred to a Centricon-10 concentrator. Samples were immediately centrifuged at 5000*g* for 1 h at 5 °C and stored at −40 °C prior to HPLC analysis within 12 h. Control experiments showed that 98 ± 2% of DHAP and NADH was recovered. NADH is known to undergo acid-catalyzed hydrolysis of the dihydropyridine ring (Warburg et al., 1935), and mixtures of NADH/NAD$^+$ undergo a self-exchange reaction at elevated temperatures (Powell & Bruice, 1983). These side reactions are not expected to contribute significantly at the pHs and temperatures used in these experiments (Cha et al., 1989; Grant & Klinman, 1989).

Secondary Kinetic Isotope Effects. Dual-Label Experiments. For dual-label experiments, tritiated DHAP (0.6−1.2 mM; 0.15−0.72 Ci/mol) was mixed with [^{32}P]NAD$^+$ (1.5−1.75 mM; 0.11−0.36 Ci/mol, ^{3}H/^{32}P = 3−0.35), sodium arsenate (6 mM), EDTA (5 mM), and 160 units of GAPDH in 50 mM TEA, pL 7.5. To initiate the reaction, 2.5 units of TIM were added. For t_∞ time points, 0.6−0.9 mM [1-^{3}H]DHAP, 160 units of GAPDH, and 1700 units of TIM were used. Aliquots were quenched with CHAP,

centrifuged on a Centricon-10 concentrator, and stored at −40 °C prior to HPLC analysis within 12 h.

HPLC analyses were performed using a 5 × 50 mm PL-SAX strong anion exchange column from Polymer Laboratories, Inc. (Amherst, MA). For later experiments two columns in series were used. Samples containing tritiated water, [^{32}P]NAD$^+$, [1-^{3}H]DHAP, and [^{3}H]NADH or [^{32}P,^{3}H]-NADH were eluted at a flow rate of 1.0 mL/min, with a gradient (0−0.5 min, 4% B, 0% C; 0.5−15 min, 4% B, 50% C; 15−25 min, 4% B, 100% C) where eluent A was 50 mM TEA HCl, pH 7.1, eluent B was acetonitrile, and eluent C was 50 mM TEA HCl, pH 7.1, containing 0.1 M NaCl. The retention times of the analytes were as follows: NAD$^+$(3.5 min), DHAP (14 min), and NADH (18 min). For infinity time points, an additional tritium-labeled peak (∼4%) was detected at 28 min corresponding to 3-phosphoglycerate. The absorbance at 340 nm was monitored, and fractions (50 × 0.5 mL) were analyzed for ^{3}H or ^{3}H/^{32}P ratios by liquid scintillation counting. Each fraction was counted for 15 min, and appropriate corrections for the half-life of ^{32}P and background counts were made. The peak area at 340 nm was found to be linearly dependent on NADH concentration.

Calculation of Kinetic Isotope Effects. Kinetic isotope effects k_L/k_T, where L = H or D, were calculated from the specific activitiy (R = ^{3}H Ci/mol of NADH) or the isotope ratios (R = ^{3}H/^{32}P for NADH) and fractional conversions, f, according to the following equation (Melander & Saunders, 1980):

$$R/R_\infty = \frac{1-(1-f)^{k_T/k_H}}{f} \qquad (1)$$

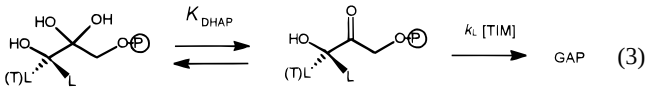
where the fractional conversion was determined from the absorbance change at 375 nm for single-label experiments. For the double-label experiments, the fractional conversion was determined from the following equation:

$$f = \left(\frac{[^{32}\text{P}]\text{NADH}}{[^{32}\text{P}]\text{NADH} + [^{32}\text{P}]\text{NAD}^+}\right)\frac{[\text{NAD}^+]_0}{[\text{DHAP}]_0} \qquad (2)$$

where [^{32}P]NADH and [^{32}P]NAD are the radioactivity for fractions containing NADH and NAD$^+$, respectively. There was generally good agreement (±1%) between fractional conversions determined by ΔA_{375} and those determined by eq 2. This provides support for the dual-label approach for determining competitive isotope effects and serves as a check on possible sources of systematic errors. The TIM reaction was shown to be rate-limiting at fractional extents of reaction f <0.75. For example, doubling the concentration of TIM had no effect on the observed isotope effect. The rate was dependent on [NAD$^+$] up to 0.3 mM (data not shown), so for f >0.75 the coupling enzyme reaction becomes partially rate-limiting and these data were not used in calculating the isotope effect. The k_H/k_T isotope effects determined from the absolute specific activity of NADH show good agreement with those calculated using ^{3}H/^{32}P ratios using independently synthesized substrates.

A possible source of error in the k_D/k_T isotope effects with [1(*R,S*)-^{2}H,^{3}H]DHAP (**3**) as the substrate in D_2O comes from the fact that the substrate is incompletely deuterated (Grant & Klinman, 1989; Rucker et al., 1992). The TOD used to synthesize **3** contained 2% HOD, and the starting [1(*R,S*)-^{2}H]DHAP contained <5% ^{1}H as measured by 500 MHz ^{1}H NMR spectroscopy. As discussed in detail by Grant and Klinman (1989), the ^{1}H contamination leads to an artificially high value for the D/T isotope effect and a systematic decrease in the D/T isotope effect with increasing fractional conversion as the protium-containing substrate is preferentially consumed. For the D/T isotope effects using [1(*R,S*)-^{2}H,^{3}H]DHAP (**3**) in D_2O, a correction for protium contamination was applied as described by Rucker et al. (1992) assuming a 2% contamination. Larger corrections give a sharp decrease in the D/T isotope effect with inverse isotope effects at even moderate fractions of reaction.

Correction for β-Deuterium Isotope Effect. DHAP exists in aqueous solution as a mixture of keto and hydrate species (Albery & Knowles, 1976b; Blacklow et al., 1988):



(3)

The observed isotope effect was corrected for the β-equilibrium isotope effect as follows. A rapid equilibrium approximation of eq 3 gives the following equation for the second-order rate constant for the TIM-catalyzed conversion of DHAP to GAP:

$$k_L = (k_L)_{obs}(1 + K_{DHAP}^{-1}) \qquad (4)$$

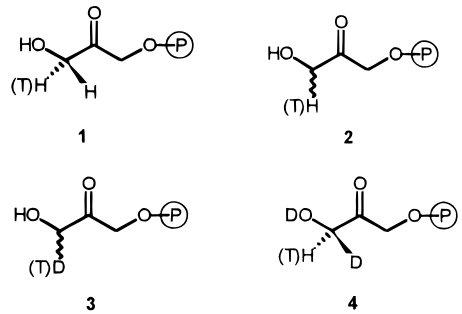
where K_{DHAP} = 2.1 in H_2O at 30 °C (Blacklow et al., 1988) and K_{DHAP} = 1.83 in D_2O at 30 °C and 50 mM TEA HCl. The observed kinetic isotope effect, $(k_H/k_T)_{obs}$, was corrected according to:

$$k_L/k_T = \Phi\left(\frac{1+K_{DHAP}}{1+K_{DHAP}\Phi}\right)(k_L/k_T)_{obs} \qquad (5)$$

where the equilibrium fractionation factor Φ is defined as $\Phi = K^T_{DHAP}/K^L_{DHAP}$. The equilibrium fractionation factor can be estimated from the β-deuterium isotope effect for ketone hydration of 0.83 (three deuteriums; Alvarez et al., 1991) and the Swain−Schaad relationship (Swain et al., 1958): $\Phi = (0.83)^{1.44/3} = 0.91$ for H/T substrates and $\Phi = (0.83)^{0.44/3} = 0.97$ for D/T substrates.

RESULTS

We have determined secondary k_H/k_T and k_D/k_T isotope effects in H_2O and D_2O for the TIM-catalyzed conversion of DHAP to GAP using four isotopically labeled substrates (indicated by structures **1**−**4**).



Compounds **1**−**4** were synthesized using *pro-S* specific aldolase and *pro-R* specific triosephosphate isomerase from yeast (Rose, 1958; Rieder & Rose, 1959) to selectively

Table 1: Summary of Secondary k_H/k_T and k_D/k_T Isotope Effects for the Triosephosphate Isomerase-Catalyzed Conversion of Dihydroxyacetone Phosphate to D-Glyceraldehyde 3-Phosphate[a]

		H/T kinetic isotope effect		D/T kinetic isotope effect	
substrate	solvent	$(k_H/k_T)_{obs}$	k_H/k_T[b]	$(k_H/k_T)_{obs}$	k_D/k_T[b]
1, 2	H_2O	1.09 ± 0.04	1.06 ± 0.04		
3	D_2O			1.067 ± 0.015	1.055 ± 0.015
4	D_2O	1.28 ± 0.03	1.27 ± 0.03		

[a] All experiments were conducted at 30 °C in 50 mM triethanolamine buffer, pL 7.5. [b] Corrected for the β-equilibrium isotope effect for hydration of DHAP using eq 5, as described in the Materials and Methods.

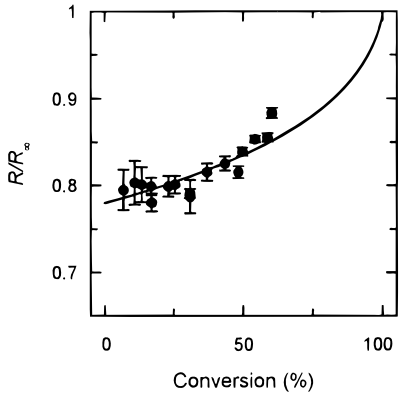
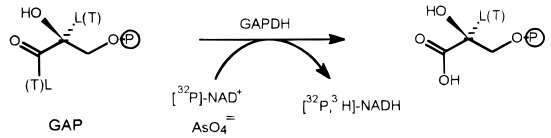


FIGURE 2: Plot of R/R_∞ as a function of percent reaction for [1(*R*)-^{2}H,1(*S*)-^{3}H]DHAP (**4**) in D_2O. The experimental data are shown as closed circles with errors of one standard deviation. The solid line is the best fit to eq 1 with $k_H/k_T = 1.28 \pm 0.03$.

exchange the C-1 proton of DHAP, as described in the Materials and Methods. Isotope effects for the TIM-catalyzed conversion of DHAP to GAP (eq 3) were determined competitively by trapping the product GAP with GAPDH/NAD^+ in the presence of arsenate ion (eq 6) (Nickbarg & Knowles, 1988).



(6)

TIM stereospecifically cleaves the 1-*pro R* proton so the amount of tritium transferred from the *pro-S* position of DHAP to NAD^+ provides a measure of the discrimination of tritium vs protium (or deuterium) in the secondary position of DHAP. [^{32}P]NAD^+ serves as a remote label to monitor the fractional conversion of the reaction of the C-H or C-D compound that is present in stoichiometric amounts. This technique is inherently more precise than noncompetitive, independent measures of the H and D rate constants that are used to determine H/D isotope effects. Isotope effects were calculated according to eq 1 from isotopic ratios and fractional conversions summarized in Tables S1 and S2.[3] Figure 2 shows a plot of the data according to eq 1 for [1(*R*)-^{2}H,1(*S*)-^{3}H]DHAP (**4**) in D_2O that corresponds to an observed isotope effect $k_H/k_T = 1.28 \pm 0.03$.

DHAP exists as an equilibrium mixture of keto and hydrate forms in aqueous solution as shown in eq 3. The change in hybridization at the C-2 carbon upon going from the hydrate to DHAP gives rise to a normal β-equilibrium isotope effect

[3] See paragraph concerning supporting information at the end of this article.

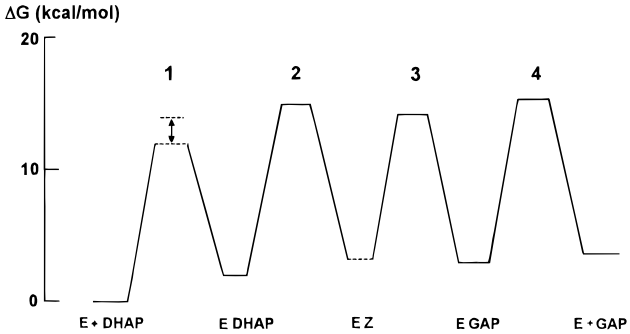


FIGURE 3: Free energy profile for the reaction catalyzed by TIM with a standard state of 40 μM. The dashed lines represent limits on those species that may be kinetically insignificant.

Scheme 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EZ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P$$

($\Phi_{DHAP} < 1$) that is independent of the TIM-catalyzed conversion of DHAP to GAP. The secondary H/T and D/T isotope effects were corrected for this β-equilibrium isotope effect as described in the Materials and Methods. Table 1 summarizes the observed and corrected H/T and D/T secondary isotope effects.

DISCUSSION

The Free Energy Profile of TIM in H_2O. Using the model of Scheme 1, the free energy profile for the TIM-catalyzed conversion of DHAP (S) to GAP (P) via an enediol(ate) intermediate (EZ) has been previously determined at 30 °C, pH 7.5, by the elegant isotope exchange studies of Albery, Knowles, and their colleagues (Albery & Knowles, 1976a–c; Leadlay et al., 1976; Nickbarg & Knowles, 1988).

The profile for the all-proton reaction in H_2O shows four energy barriers, all of which are kinetically significant (Figure 3). Although the transition state for association of DHAP with TIM is lower in energy than the three subsequent steps, the rate constant for dissociation of enzyme-bound DHAP from the ES complex (k_{-1}) is not substantially smaller than the rate constant for ionization of enzyme-bound DHAP, k_2. Since the transition-state energies of each of these steps in Scheme 1 are comparable, no one step is cleanly rate-limiting. The physical steps serve to lower the magnitude of the observed secondary isotope effects relative to the intrinsic isotope effect for the enolization steps for substrates with protium in the 1-*pro-R* position in H_2O (Albery & Knowles, 1976a). This is consistent with the small magnitude of the observed k_H/k_T isotope effect in H_2O, $k_H/k_T = 1.06 \pm 0.04$.

Estimation of the Intrinsic Isotope Effect. In order to interpret the secondary k_H/k_T isotope effect in H_2O, we derive

Table 2: Evaluation of the Intrinsic Secondary k_2^H/k_2^T Isotope Effect

	M^{-1} s^{-1}		
	$k_1 = 4 \times 10^8$	$k_1 = 10^8$	$k_1 = 10^7$
$\alpha = k_2/k_{-1}$[a]	0.003	0.02	0.5
θ	1.2	2.9	4.6
k_2^H/k_2^T[b]	>1.06	1.21	<1.48

[a] Limiting values for the partition ratio α were calculated according to eq A.3 as described in the Appendix using limiting values for k_1 and a mean value for the partition ratio $\theta = 2.9 \pm 1.7$ for yeast TIM (Nickbarg & Knowles, 1988). [b] Values of the intrinsic secondary isotope effect, k_2^H/k_2^T, were calculated by correcting the H/T isotope effect for **1** in H_2O (Table 1) using eq A.4 with limiting values for the partition ratios α and θ.

limiting values of the intrinsic isotope effect, k_2^H/k_2^T, in the Appendix, following the treatment of Scheme 1 by Albery and Knowles (1976a). This yields the "best value" for the intrinsic isotope effect $k_2^H/k_2^T = 1.21$, with a range $k_2^H/k_2^T = 1.06-1.48$ as summarized in Table 2. This value must be interpreted conservatively because errors in the partition ratios are ±60% or more. Nevertheless, the results show the expected normal isotope effect for rehybridization of an sp^3 C-H bond in DHAP to an sp^2-like enediol(ate) intermediate.

Further support comes from H/T and D/T experiments with deuterium in the *pro-R* position in D_2O. These experiments were carried out in order to slow the chemical steps (k_2 and k_3 in Scheme 1), relative to the substrate binding and product release steps, and therefore simplify the interpretation of the isotope effects. The secondary k_H/k_T isotope effect for the [1(*R*)-^{2}H]-labeled substrate is $k_H/k_T = 1.27 \pm 0.03$ (Table 1), and the k_D/k_T isotope effect corresponds to $k_H/k_T = (k_D/k_T)^{3.3} = 1.17 \pm 0.08$.

The following points suggest that deuterium substitution of the substrate in the *pro-R* position, cf. substrates **3** and **4**, in D_2O reduces the magnitude of the partition ratios in eq A.4 (Appendix) to the point where the intrinsic secondary H/T and D/T isotope effects are completely expressed: (1) The observed Swain−Schaad exponent $y = \ln(k_H/k_T)/\ln(k_D/k_T) = 4.4 \pm 1.3$ is consistent with essentially complete rate limitation by the enolization step, k_2 (Albery & Knowles, 1976a). Swain−Schaad exponents that exceed the classically allowed limit $y = 3.3$ are also consistent with coupled motion of the *pro-R* and *pro-S* hydrogens and tunneling (Cha et al., 1989). (2) The initial rate of reaction of [1(*R*)-^{2}H]DHAP shows a deuterium kinetic isotope effect on k_{cat}/K_M in H_2O, $^D(k_{cat}/K_M) = 3.4$ (Nickbarg & Knowles, 1988). This suggests that substrates with deuterium in the *pro-R* position do not react at the diffusion limit and are limited by chemical steps. (3) The solvent isotope effect on k_{cat} with GAP as the substrate is 1.5 ± 0.2 for a mutant isomerase where the active-site glutamate-165 has been replaced by aspartate (Raines et al., 1986). For this mutant, the enolization steps are about 1000-fold slower, so this solvent isotope effect suggests that the hydron transfer steps 2 and 3 are slowed in D_2O (Albery & Gelles, 1982). Taken together, these data suggest that secondary tritium isotope effects for the reaction of [1(*R*)-^{2}H]-labeled DHAP in D_2O will approach the intrinsic isotope effect for the enolization reaction (k_2 in Scheme 1).[4]

Equilibrium H/T Isotope Effect for Enolization. The equilibrium isotope effect $K_H/K_T = 1.11-1.12$ for enolization of DHAP was calculated at the HF/3−21+G* level ($k_H/k_T = 1.12$; Alston, 1995) or from the data reported by Cleland (1987).[5] It should be noted that much larger equilibrium isotope effects are observed for enolization of α-methyl ketones and acetaldehyde due to hyperconjugation effects when the heavy isotope is *syn* to the carbonyl group (Alston, 1995; Alston et al., 1996). Large equilibrium isotope effects are unlikely in the TIM reaction since stereochemical and crystallographic evidence for a *cis*-enediol(ate) intermediate (Rose, 1982; Davenport et al., 1991) requires the C-1 OH group to be *syn* to the C-2 carbonyl.

Coupled Motion and Hydrogen Tunneling. The secondary H/T and D/T isotope effects summarized in Tables 1 and 2 are normal ($k_H/k_T > 1$) and large. A similar large secondary isotope effect $k_H/k_T = 1.28$ has been observed with yeast aldolase, an enzyme that carries out proton transfers from DHAP with opposite stereochemistry (Biellmann et al., 1969). These isotope effects exceed the maximum equilibrium isotope effect for ionization of DHAP. In addition, for the TIM reaction the Swain−Schaad exponent relating D/T and H/T secondary isotope effects $y = 4.4 \pm 1.3$ is larger than the semiclassical upper limit of 3.3, although there is a considerable uncertainty due to propogation of errors. These results are similar to observations in hydrogen transfer reactions where coupled motion of the primary and secondary hydrogens and tunneling have been invoked in order to account for unusually large secondary isotope effects and small, normal primary isotope effects (Huskey & Schowen, 1983; Cha et al., 1989). However, an unusual feature of the TIM reaction is that large secondary isotope effects are observed even when the a deuterium atom is transferred. Deuterium tunneling in enzyme reactions has been demonstrated in the plasma amine oxidase reaction (Grant & Klinman, 1989) where unusually large primary kinetic isotope effects are observed.

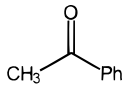
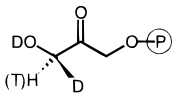
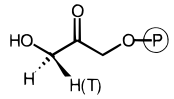
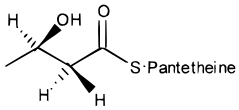
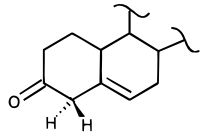
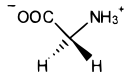
There is some evidence for proton tunneling in nonenzymatic enolization reactions, based on ratios of Arrhenius preexponential factors that are substantially less than the semiclassical lower limit (Jones, 1969). However, coupled motion of both protons does not appear to be a requirement based on the small secondary isotope effects observed for enolization reactions in solution (Alston et al., 1996). Similarly, deviations from the Swain−Schaad exponent $y = 1.44$ relating H/D and H/T isotope effects are generally small (Caldin, 1969; Jones, 1969), although large deviations due to hydrogen tunneling are not expected.

The Nature of the Transition State for Enzymatic Enolization Reactions. We would like to know how enzymes might alter the transition-state structure for enolization reactions relative to the corresponding reactions in solution. It is well established that a substantial fraction of the barrier for ionization of C-H acids in solution and the gas phase is the lag in resonance delocalization of charge relative to C-H

[4] In principle, there may be a small contribution to the secondary isotope effect from the enediol(ate) to GAP isomerization step, k_3. However, this isotope effect is expected to be small and slightly inverse (Cleland, 1987), as the C-1 carbon remains sp^2 hybridized in both species.

[5] The estimated deuterium fractionation factor for the *cis*-enediolate is (0.78)(1.18) = 0.91 based on phosphoenolpyruvate and the effect of an α-OH substitutent adjacent to the C-L bond. This yields an equilibrium isotope effect = $(0.99/0.91)^{1.44} = 1.11$ for ionization of DHAP.

Table 3: Secondary Kinetic and Equilibrium Isotope Effects for Proton Transfer Reactions

Catalyst	Substrate	k_H / k_T	K_H / K_T	Reference
OH^-		1.09 ± 0.03 [a]	1.18-1.33	*b*
Triosephosphate Isomerase		1.27 ± 0.03	1.12	this work
Yeast Aldolase		1.28	1.12 [c]	*d*
Enoyl-CoA Hydratase		1.18 ± 0.02 [a]	1.12	*e*
3-Oxo-Δ^5-steroid Isomerase		1.16 ± 0.02 [a]	1.18	*f*
D-Amino Acid Oxidase		0.99 - 1.02	1.13	*g*

[a] Derived from $k_H/k_T = (k_H/k_D)^{1.44}$. [b] Alston et al. (1996). [c] Cleland (1987) has estimated $K_H/K_T = 1.16$. [d] Biellman et al. (1969); isotope effect at pH 9 where committment factors are negligible. [e] Bahnson and Anderson (1989). [f] Xue et al. (1990). [g] Denu and Fitzpatrick (1994). The equlibrium isotope effect K_H/K_T is calculated (Cleland, 1987) from the deuterium fractionation factors for glycine (1.13/1.15 = 0.98) and $C{=}C[H]NH_3^+$ ((0.81)(1.15) = 0.93) using the Swain−Schaad relationship: $K_H/K_T = (0.98/0.93)^{1.44} = 1.13$.

transfer in the transition state (Bernasconi, 1987, 1992). We have argued that the small secondary isotope effects observed in nonenzymatic enolization and ketonization reactions reflect this kinetic barrier for charge reorganization (Alston et al., 1996). If coupled motion and tunneling are important requirements for overcoming this lag in resonance delocalization, then large H/T isotope effects might be expected to be a general feature of enzymatic enolization reactions. Table 3 summarizes secondary H/T isotope effects for hydroxide ion-catalyzed and enzyme-catalyzed enolization reactions. In contrast to the non-enzyme-catalyzed enolization reaction, enzymatic enolization reactions display large secondary k_H/k_T or k_H/k_D isotope effects that approach or exceed the maximum equlibrium isotope effect.[6] The H/T isotope effect in the reductive half-reaction of D-amino acid oxidase (Table 3, entry 6) is close to unity, but the evidence which suggests that this reaction proceeds via a carbanion mechanism has been challenged based on the absence of a putative general base catalyst in the X-ray structure (Mattevi et al., 1996). Overall, the data are consistent with the supposition that large H/D or H/T isotope effects that approach or exceed the equilibrium isotope effects are the rule, rather than the exception, in enzymatic enolization reactions. In this context, the data reported here lend some qualitative support to the conclusion of Gerlt and Gassmann (1993b) that enzymatic transition states for enolization reactions must be late (i.e., enol-like) in order to take complete advantage of the stabilization provided by "short, strong hydrogen bonds" that lower the intrinsic barrier of the reaction.

Proton Tunneling in Enzyme Reactions. The data reported here add to a growing list of examples of tunneling in enzymatic hydron (Xue et al., 1990; Grant & Klinman, 1989; Rucker et al., 1992) and hydride transfer reactions (Huskey & Schowen, 1983; Cha et al., 1989). There are several considerations that suggest that tunneling may be more important for enzyme than non-enzyme-catalyzed enolization reactions. It has been suggested that proton transfer in solution may be coupled with solvent reorientation and this increases the effective mass of the proton (Caldin, 1969; Kurz & Kurz, 1972; Bernasconi, 1992). Water is restricted from the active site of many enzymes catalyzing proton transfers to and from carbon (Gilbert, 1980). This may lead to a smaller effective mass and therefore higher tunneling probabilities on enzymes. The simple electrostatic model for proton tunneling proposed by Bell (1980), as well as more sophisticated models of resonance tunneling (Dogonadze et al., 1977; Sarai & DeVault, 1986; Bruno & Bialek, 1992), require low dielectric media and equilibrium constants close

[6] It should be noted that secondary isotope effects in nonenzymatic reactions were determined for endergonic reactions. Since the corresponding enzyme reactions are likely to be near thermoneutral (Albery & Knowles, 1976c) or less endergonic (Gerlt & Gassman, 1993b), a simple Hammond postulate argument would require even smaller H/T secondary isotope effects for enzyme reactions, in the absence of coupled motion and tunneling.

to unity for efficient tunneling; both situations are likely to occur on enzyme surfaces (Albery & Knowles, 1976c; Warshel & Russell, 1984; Rucker et al., 1992). It has been proposed that enzyme reaction barriers may be compressed relative to those in solution, resulting in an alteration in the barrier width as well as height on enzyme surfaces (Rodgers et al., 1982; Cha et al., 1989; Bruno & Bialek, 1992). In the TIM reaction, the active-site base moves some 2−3 Å upon formation of the ES complex, and the short (2.6 Å) O−H−N hydrogen bond between the O_γ of E165 and the nitrogen of the phosphoglycolohydroxamate inhibitor (Davenport et al., 1991) suggests that TIM can sample conformations in the ES complex that correspond to short reaction barriers. However, there are ony a few enzymes for which there is direct evidence for catalysis by compression (Rodgers et al., 1982; Frey et al., 1994). It is not known how enzymes can provide the large force constants that are required for small distortions of reaction barriers (Jencks,1987; Kraut, 1988; Williams, 1993).

ACKNOWLEDGMENT

We gratefully acknowledge the support and encouragement of Professor Frank Millett.

APPENDIX: DERIVATION OF LIMITING VALUES FOR THE INTRINSIC H/T ISOTOPE EFFECT IN H_2O

The steady-state treatment of Scheme 1 yields an expression (Albery & Knowles, 1976a) for the kinetic significance of the microscopic rate constants in terms of $(k_{cat}/K_M)^{-1}$:

$$A_1 = K_M/k_{cat} = \frac{(1+\alpha)(1+\theta)}{ak_1} \qquad (A.1)$$

where $\alpha = k_2/k_{-1}$ is a partition ratio describing the forward commitment to catalysis from ES and θ is an effective partition ratio describing the fate of the intermediate EZ in Scheme 1 forward and backward:

$$\theta = \frac{k_{-2}}{[1+(k_2/k_{-1})]}\frac{[1+(k_{-3}/k_4)]}{k_3} \qquad (A.2)$$

A large value of θ means that EZ goes to S more than to P, and a small value of θ means that EZ goes to P more than S.

Eq A.1 can be solved for the partition ratio α:

$$\alpha = \left[\frac{A_1k_1}{1+\theta} - 1\right]^{-1} \qquad (A.3)$$

in terms of two measured quantities, A_1 and θ, and estimates of limiting values for the second-order rate constant for association of DHAP with the enzyme: $4 \times 10^8\ M^{-1}\ s^{-1} \geq k_1 \geq 10^7\ M^{-1}\ s^{-1}$. Although this rate constant has not been measured directly, the second-order rate constant $k_{cat}/K_M = 3.7 \times 10^8\ M^{-1}\ s^{-1}$ in the reverse direction is diffusion-controlled (Blacklow et al., 1988), i.e., limited by the rate at which GAP binds to the enzyme. Diffusion is a physical, not a chemical, process and should not depend on the relative arrangements of bonds in GAP and DHAP (aldehyde−alcohol vs keto−alcohol) so this places an *upper* limit on the rate constant for association of DHAP with the enzyme: $k_1 \leq 4 \times 10^8\ M^{-1}\ s^{-1}$. These limits are also consistent with the Brownian dynamics calculations of Luty et al. (1993). Combining these estimates with $\theta = 2.9 \pm 1.7$ (Nickbarg & Knowles, 1988) and $k_{cat}/K_M = 1/A_1 = 5 \times 10^5\ M^{-1}\ s^{-1}$ yields limiting values for the partition ratio: $0.003 \leq \alpha \leq 0.5$.[7]

From eqs 1.8 and 4.6 of Albery and Knowles (1976a) we obtain an expression describing the observed secondary isotope effect in terms of the intrinsic isotope effect k_2^H/k_2^T for the enolization step:

$$A_3/A_1 = (k_H/k_T)_{obs} = (k_2^H/k_2^T)\left(\frac{1+\alpha'}{1+\alpha}\right)\left(\frac{1+\theta'}{1+\theta}\right) \qquad (A.4)$$

where the primes indicate substitution by tritium in the *pro-S* position.[8]

Two simplifying assumptions are required to establish limiting values for the intrinsic isotope effect for the enolization reaction: (1) There is no isotope effect on the rate constant for association of DHAP with the enzyme, i.e., the fractionation factor $\Phi_1 \sim 1$. This assumption is supported by (a) the small isotope effect (<3%) for the diffusion of TOH in H_2O and TOD in D_2O (Weingärtner, 1982) and (b) the absence of a significant β-deuterium isotope effect (<4%) on the protonation of acetone (Alston et al., 1996) that serves as a limiting model for the polarized ES complex in Figure 1. (2) Following Raines et al. (1986), since the ratios α/α' and θ/θ' cannot be more than the intrinsic isotope effect on one of the enolization steps, these ratios are not expected to exceed ca. 1.3 (Alston et al., 1996).

SUPPORTING INFORMATION AVAILABLE

Figure S1 showing a representative separation of tritiated water, [^{32}P]NAD$^+$, [1(*R,S*)-^{3}H,^{2}H]DHAP, and [^{32}P,^{3}H]NADH by anion exchange chromatography, Table S1 summarizing fractional extents of reaction and isotopic ratios for [1(*S*)-^{3}H]DHAP (**1**) and [1(*R,S*)-^{3}H]DHAP (**2**) in water, and Table S2 giving the corresponding data for [1(*R,S*)-^{3}H,^{2}H]DHAP (**3**) and [1(*R*)-^{2}H, 1(*S*)-^{3}H] DHAP (**4**) in deuterium oxide (5 pages). Ordering information is given on any current masthead page.

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[7] The partition ratio α is probably closer to the lower limit based on the fact that the primary kinetic isotope effects $^D(k_{cat}/K_M)$ and $^Dk_{cat}$ are equal, within experimental error (Nickbarg & Knowles, 1988; cf. eq 1.8 of Albery and Knowles (1976a)). This requires that the enolization step be cleanly rate-limiting, relative to the k_1 step, even though the proton transfer step k_2 is not rate-limiting for the overall reaction since these initial rate isotope effects reflect only the steps entering the enediol(ate) pool.

[8] Note that since there is no isotopic exchange at EZ, $A_5 = 1$ in eq 4.6 of Albery and Knowles (1976a) and this means that A_3 reduces to the primed counterpart of A_1.

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