Primary and Secondary Kinetic Isotope Effects as Probes of the Mechanism of Yeast Enolase

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ABSTRACT: Enolase catalyzes the interconversion of 2-phosphoglycerate and phosphoenolpyruvate. Kinetic isotope effects have been used to determine whether abstraction of the proton from C-2 and loss of hydroxide from C-3 of 2-phosphoglycerate occur in a concerted reaction or as sequential processes and whether these steps are kinetically significant for the enolase-catalyzed reaction. Enolase exhibits a significant primary deuterium isotope effect, as well as catalyzing the relatively rapid exchange of the C-2 proton with solvent water. Secondary C-3 deuterium isotope effects are also reported, both when the C-2 carbon carries a hydrogen and when this center is deuterated. These results provide information about the kinetic significance and timing of the transition state(s) associated with the loss of H^+ and OH^-. Strong evidence has been presented for a stepwise mechanism where both the rate of proton abstraction and one or both of the later transition states, *i.e.*, those associated with hydroxide loss and product release, limit the overall reaction rate. If a concerted reaction were to be invoked, the presence of a small secondary ^2H isotope effect in combination with the observed rate of exchange of the C-2 proton require the intrinsic secondary ^2H kinetic isotope effect to be effectively unity. For the concerted mechanism, an intrinsic effect of unity would be consistent only with an extremely asymmetric transition state that is dominated by C–H bond cleavage.

Enolase catalyzes the reversible dehydration of 2-phospho-D-glycerate (2-PGA) to phosphoenolpyruvate (PEP). In the *anti* elimination of water from 2-PGA, two bonds are broken, the carbon–hydrogen bond at C-2 and the carbon–oxygen bond at C-3. The incorporation of ^2H at C-2 or of ^{18}O at C-3 of 2-PGA affects the rate at which the substrate proceeds through the corresponding transition state(s) of the catalyzed reaction. The effect of these substitutions on the overall rate of reaction depends on the kinetic significance of the associated transition state(s) relative to the other steps in the reaction pathway.

Enolase is known to exhibit a significant primary deuterium kinetic isotope effect that is dependent on both the pH and Mg(II) concentration (Shen & Westhead, 1973; Anderson, 1981). A primary ^{18}O effect of 1.02–1.03 has also been reported. At pH values above 7.8, enolase also catalyzes the exchange of the proton on C-2 with the protons of the solvent water at a rate that is faster than substrate turnover. At lower pH values, however, in the range where a sizable primary isotope effect is seen, the exchange of this proton is insignificant (Dinovo & Boyer, 1971).

The presence of a primary ^2H effect, an ^{18}O effect, and the reported exchange of the C-2 proton do not, however, permit the determination of whether the C–H and C–O bond cleavages occur in a single step or in two separate steps with a distinct intermediate, *i.e.*, whether the reaction is concerted or stepwise. Although the reported data have been interpreted as being *consistent* with a stepwise carbanionic mechanism, PEP formation by any mechanism could produce the observed results if the hydroxide fragment were sequestered more tightly than the proton in the active site of the enzyme. That the release of one or more products may limit the overall rate of reaction is well known and may derive from the evolutionary pressure that accelerates chemical steps until these steps are just slightly faster than those involving product release (Albery & Knowles, 1977).

Other isotope studies have also been interpreted as favoring a stepwise mechanism; yet, none of the evidence is unequivocal. Dinovo and Boyer (1971) monitored ^2H and ^{18}O exchange into the positions that equilibrate with solvent H_2O. The ^2H exchange was always faster than the ^{18}O exchange, leading to the proposal that the C–H bond at C-2 was cleaved more readily and more often than the C–O bond at C-3. These experiments were, however, performed at high Mg(II) concentrations (25 mM), where substrate and product release are rate limiting. Consequently, this experiment only reports on the relative ease of exchange, not on the timing of the bond breaking processes. In fumarase, for example, ^{18}O exchange is more rapid than ^2H exchange even though the reaction is presumed to proceed via a carbanion intermediate (Blanchard & Cleland, 1980; Porter & Bright, 1980).

Further support that the enolase-catalyzed reaction proceeds via a stepwise mechanism is provided by the enolase-catalyzed elimination of HCl and HF from 3-chloro- and 3-fluoro-2-PGA, respectively. These eliminations have been shown to proceed via an anionic intermediate due to (a), the presence of only a small secondary deuterium isotope effect (<2%) for [3,3-$^2H_2$]chloro(fluoro)-2-PGA and (b), an inverse leaving group effect, the elimination of HF being faster than the elimination of HCl (Stubbe & Abeles, 1980). Likewise, enolase has been shown to catalyze the stepwise elimination of H_2O from the phosphonate analogue of 2-PGA (Anderson & Cleland, 1990). In all three of these alternate substrates, however, the acidity of the α-proton is increased significantly. Thus, the observed anionic intermediates could reflect a change in mechanism and do not necessarily signify that the enolase-catalyzed elimination of H_2O from its natural substrate also occurs in this manner.

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More compelling evidence for the stepwise mechanism comes from the inhibitor studies of Anderson, Weiss, and Cleland (1990). These authors prepared and tested analogues of the *aci*-form of the proposed anionic intermediate. A number of these compounds were found to be good inhibitors of the enolase-catalyzed reaction, with the tightest binding inhibitor, phosphonoacetohydroxymate, having a K_i of 15 pM.

We report in this paper determinations of the primary deuterium, primary ^{18}O, and secondary deuterium kinetic isotope effects for the enolase-catalyzed reaction. These studies, using the actual substrates under conditions where the bond-breaking processes are rate-limiting, illuminate the mechanism of the reaction, specifically with regard to the timing and kinetic significance of the transition states for the abstraction of the C-2 proton and the loss of hydroxide from C-3 of 2-PGA. These studies are timely as the structure of the active site environment of enolase is becoming better defined (Lebioda & Stec, 1991).

MATERIALS AND METHODS

Enzymes. Yeast enolase was from Sigma Chemical Co. (St. Louis, MO). Pyruvate kinase, lactate dehydrogenase, hexokinase, and alkaline phosphatase were from Boehringer Mannheim Biochemicals (Indianapolis, IN). PEP synthetase was isolated from *Escherichia coli* strain B and was purified according to the procedure of Cooper and Kornberg (1969) as modified by Berman and Cohn (1970).

Chemicals. 2-Phosphoglycerate, phosphoenolpyruvate, pyruvate, ADP, and NADH were from Sigma. Pentafluorobenzyl bromide was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Deuterium oxide was from Cambridge Isotope Laboratories (Woburn, MA; 99.9% atom % excess) or Merck, Sharp and Dohme Isotopes (Montreal, Canada; 99.96 atom % excess). $H_2{}^{18}O$ was from Cambridge Isotope Laboratories (95 atom % excess) or ISOTEC, Inc. (Miamisburg, OH; 97.2 atom % excess).

Primary Deuterium Equilibrium Isotope Effect. The primary deuterium equilibrium isotope effect was determined at 25 °C and at pH values of 5.55, 6.65, and 7.70. The equilibrium isotope effects were determined in either 128 mM MES[1] buffer or 140 mM MOPS buffer containing $MgSO_4$ (500 μM) and EDTA (10 μM). The equilibrium values were obtained by observing the ultraviolet absorbance of PEP before and after equilibration with enolase. Extinction coefficients were determined experimentally in both 90% (v/v) D_2O and in H_2O at each of the three pH values. Values for K_{eq} were determined in 90% (v/v) D_2O and in H_2O. K_{eq} values in 100% D_2O were extrapolated from the experimental data assuming a linear relationship between K_{eq} and the mol fraction of D_2O. The equilibrium isotope effect was calculated using these experimentally-determined values of ${}^{H}K_{eq}$ and ${}^{D}K_{eq}$ and a correction factor of 0.945, which is needed to account for differences in the fractionation factors of HOD and D_2O (Cleland, 1980).

Synthesis of 2-Phospho[2-^{2}H]glycerate. PEP was converted enzymatically to 2-phospho[2-^{2}H]glycerate ([2-^{2}H]-2-PGA). PEP (140 mg, 0.68 mmol) was added to 100 mM MES buffer (10 mL) containing $MgSO_4$ (1.5 mM) and EDTA (10 μM). The pH of the solution was adjusted to pH 5.6, and the solution was lyophilized. Crystalline yeast enolase (4 mg, 200 units) and D_2O (20 mL) were added, and the solution was incubated at room temperature. The progress of the reaction was monitored by measuring the decrease in A_{236nm} due to the depletion of PEP. Once the system had reached equilibrium, the enzyme was removed by filtering the solution through a Centricon-10 concentrator (Amicon), and the solution was subjected to chromatography on a column (1.8 × 10 cm) of AG1-X8 anion-exchange resin, eluting with a linear gradient of NH_4HCO_3 (100–400 mM, 2 × 400 mL). The fractions containing 2-PGA were combined and concentrated to 20 mL. $MgSO_4$ (0.03 mmol), KCl (0.03 mmol), ADP (0.50 mmol), pyruvate kinase (60 units), and lactate dehydrogenase (40 units) were added, and the solution was titrated with NADH (48 mM) until the absorbance at 339 nm had stabilized as a function of time. This mixture was passed through a plug of charcoal, and the resulting solution was resubjected to chromatography on an AG1-X8 column (1.8 × 10 cm), eluting with NH_4HCO_3 (100–400 mM, 2 × 400 mL). The fractions containing 2-PGA were combined. The solution was titrated with LiOH to pH 9.0–9.5 and then lyophilized. The 2-PGA was precipitated from H_2O (10 mL) with MeOH (50 mL). The solid was collected and passed through Dowex-50W (K^+ form, 1.2 × 6 cm). The potassium salt of [2-^{2}H]-2-PGA was stored as a lyopholized powder at −78 °C.

^{1}H NMR (400 MHz, D_2O) 2-PGA standard: δ 3.80 (1H, dd), 3.91 (1H, dd), 4.48 (1H, m); [2-^{2}H]-2-PGA: δ 3.70 (1H, d), 3.80 (1H, d).

Primary Deuterium Isotope Effect by Direct Intermolecular Competition. Kinetic measurements for the competition experiments were made using a Uvikon 860 spectrophotometer (Kontron instruments) at 30.0 °C. The primary deuterium isotope effect for the conversion of 2-PGA to PEP was determined as follows. A mixture of [2-^{1}H]-2-PGA (1.00 mM) and [2-^{2}H]-2-PGA (0.487 mM) was incubated at 25 °C in 50 mM potassium MES buffer (15 mL), pH 6.55, containing ADP (1.86 mM), NADH (2.31 mM), $MgSO_4$ (0.5 mM), glucose (1.34 mM), hexokinase (20 units), lactate dehydrogenase (120 units), and pyruvate kinase (200 units). The reaction was initiated by the addition of enolase (2–4 units) and was followed by monitoring the decrease in A_{339nm} due to the depletion of NADH over the course of the reaction. Samples were removed after 40 and 60% completion and were quenched by the addition of Dowex-50W (H^+ form). Each solution was passed through a column (1.2 × 7 cm) of Dowex-50W (H^+ form), the eluate was neutralized with imidazole, and the neutralized solution was loaded onto a Mono-Q HPLC column. The 2-PGA was eluted using a gradient of CsCl (0–160 mM) at a flow rate of 2 mL/min. Under these conditions 2-PGA and ADP coelute. The HPLC fractions were assayed for 2-PGA, and the appropriate fractions were combined. The solutions were filtered through a plug of charcoal to remove the ADP, and the samples were then prepared for mass spectral analysis.

Preparation of Pentafluorobenzyl Glycerate from 2-PGA. The substrate samples were analyzed without further purification using a gas chromatograph–mass spectrometer. To facilitate product analysis in this manner, the phosphate group was removed as follows. The pH of the sample was adjusted to 9.0, alkaline phosphatase (25 units) was added, and the solution was allowed to incubate for 24 h at room temperature, resulting in the conversion of the 2-PGA to D-glycerate. The pH of the solution was lowered to 7.0, and the solution was concentrated under reduced pressure.

The resulting glycerate sample was dissolved in 50 mM MES (0.67 mL), pH 6.0, and the solution was diluted with

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography.

acetone (1.33 mL). α-Bromopentafluorotoluene (5 μL, 0.030 mmol) was added and the solution stirred at 55–60 °C. After 1 h, a second portion of bromopentafluorotoluene (5 μL, 0.030 mmol) was added, and the solution was stirred for an additional hour. The resulting pentafluorobenzyl glycerate was extracted from the aqueous solution into ethyl acetate (2 mL × 3), the solution was dried over anhydrous $MgSO_4$, and the resulting pentafluorobenzyl ester was concentrated under reduced pressure.

The hydroxyl groups of pentafluorobenzyl glycerate were acetylated by dissolving the sample in a mixture of acetic anhydride (0.67 mL) and pyridine (0.33 mL). The solution was stirred at 60 °C for 5–10 min and was again concentrated under reduced pressure. All samples were stored at −20 °C.

Gas-Phase Chromatography, Mass Spectral Analysis. The mass spectral analyses were performed on a Hewlett-Packard 5988A mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph. The analyses were obtained using negative chemical ionization (NCI). Samples were subjected to chemical ionization with methane as the reagent gas and a source temperature of 250 °C. The analyses were conducted in the single ion monitoring mode (SIM) to detect the isotope peaks with *m/z* values of 189, 190, and 191 (M, M + 1, M + 2).

Determination of an Exchange Rate for the C-2 Proton of [2-^{2}H]-2-PGA with Solvent H_2O. The rate of the enolase-catalyzed exchange of the C-2 proton of [2-^{2}H]-2-PGA with solvent H_2O was determined using the procedure described for the primary deuterium isotope effect. [2-^{2}H]-2-PGA (1.35 mM) was preincubated at 25 °C in 50 mM potassium MES buffer (15 mL), pH 6.55, containing ADP (1.86 mM), NADH (2.31 mM), $MgSO_4$ (0.5 mM), glucose (1.34 mM), hexokinase (30 units), lactate dehydrogenase (120 units), and pyruvate kinase (200 units). Enolase (0.5 units) was added to initiate the conversion of 2-PGA to PEP. Samples were taken after 3%, 6%, 9%, and 12% conversion to product, and the samples were processed for mass spectral analysis as described above.

Data Analysis. The concentrations of [2-^{1}H]-2-PGA and [2-^{2}H]-2-PGA present after partial conversion to product for both the competition and washout experiments were determined from the initial concentrations of 2-PGA, the extent of reaction, and the appropriate mass peak ratios. The primary deuterium kinetic isotope effect and rate of washout were determined using the data analysis graphics program KALEIDAGRAPH (Synergy Software).

Synthesis of 2-Phospho[3-^{18}O]glycerate. PEP was converted enzymatically to 2-phospho[3-^{18}O]glycerate ([3-^{18}O]-2-PGA) in the same manner as for the synthesis of [2-^{2}H]-2-PGA, with the following modifications. PEP (70 mg, 0.634 mmol) was added to 100 mM MES buffer (5 mL) containing $MgSO_4$ (1.5 mM) and EDTA (10 μM). The pH of the solution was adjusted to 5.6, and the solution was lyophilized. The resulting solid was redissolved in H_2O (3.5 mL), and this solution was diluted to 5.0 mL with $H_2{}^{18}O$. Crystalline enolase (1 mg, 50 units) was added, and the solution was allowed to incubate at room temperature. The progress of the reaction was monitored by observing the decrease in A_{236nm} due to the depletion of PEP. Once equilibrium had been attained, the [3-^{18}O]-2-PGA was purified as described for [2-^{2}H]-2-PGA.

Primary ^{18}O Isotope Effect by Intermolecular Competition. To conduct the ^{18}O competition experiments, a mixture of [3-^{16}O]-2-PGA (0.94 mM) and [3-^{18}O]-2-PGA (0.40 mM) was incubated at 25 °C in 50 mM potassium MES buffer (15 mL), pH 6.55, containing ADP (1.86 mM), NADH (2.31 mM), $MgSO_4$ (0.5 mM), glucose (1.34 mM), hexokinase (20 units), lactate dehydrogenase (120 units), and pyruvate kinase (200 units). The reaction was initiated by the addition of enolase (2–4 units) and was followed by the decrease in A_{339nm}. Samples were taken after 40% and 60% overall reaction. These samples were processed for mass spectral analysis as described.

Preparation of Trideuteriopyruvate. Sodium pyruvate (0.65 g, 0.59 mmol) was dissolved in D_2O (6.5 mL). A portion (0.5 mL) was reserved for spectroscopic analysis, and the remaining solution (6 mL) was put in a sealed glass pressure tube (7 mL capacity), which was heated to 140 °C for 45 min (Rose, 1960). The resulting deuteriopyruvate was assayed enzymatically using lactate dehydrogenase (Bergmeyer, 1974). ^{1}H NMR was used to estimate the degree of isotopic exchange by comparing the exchanged sample to the original pyruvate sample. The NMR spectrum indicated complete exchange of the C-3 protons.

Synthesis of 3,3-Dideuteriophosphoenolpyruvate. Trideuteriopyruvate was converted to 3,3-dideuteriophosphoenolpyruvate ([3,3-$^2H_2$]PEP) using PEP synthetase. Trideuteriopyruvate (0.19 mmol) was added to 100 mM Tris-HCl buffer (125 mL), pH 8.3, containing $MgCl_2$ (0.01 M) and ATP (0.01 M). A portion of PEP synthetase was added, and the solution was incubated at room temperature. The progress of the reaction was monitored by observing the loss of pyruvate. Samples (100 μL) were taken as a function of time. Each sample was added to a mixture of H_2O (0.9 mL) and 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl) (0.33 mL). The solutions were incubated at 37 °C for 10 min and then quenched with 2 M NaOH (1.67 mL), incubated for an additional 10 min, and finally filtered through a cotton plug. Under these conditions 1 μmol of pyruvate has an absorbance of 4.5 at 445 nm.

Once pyruvate depletion had ceased, the solution was filtered through charcoal, and the PEP synthetase was removed by running the solution through a column (1.2 × 6 cm) of Dowex-50W (H^+ form). PEP was isolated using an AG1-X8 column (1.8 × 12 cm), eluting with NH_4HCO_3 (50–400 mM, 2 × 400 mL). Fractions containing PEP were combined, titrated with LiOH to pH 9.0–10.0, and then lyophilized. The PEP was precipitated from H_2O (20 mL) with MeOH (100 mL). A ^{31}P NMR spectrum of the resulting solid showed a peak due to inorganic phosphate (P_i). The PEP and P_i were separated on a second AG1-X8 column (1.8 × 10 cm), eluting with NH_4HCO_3 (50–300 mM, 2 × 400 mL). A ^{1}H NMR spectral analysis of the resulting labeled PEP indicated that some back-exchange (~30%) of the C-3 protons had occurred during the conversion from pyruvate to PEP.

Synthesis of 2-Phospho-3,3-Dideuterioglycerate. 3,3-Dideuteriophosphoenolpyruvate ([3,3-$^2H_2$]PEP) was converted to 2-phospho-3,3-dideuterioglycerate ([3,3-$^2H_2$]-2-PGA) in buffered H_2O using the same procedure as that for the synthesis of [2-^{2}H]-2-PGA. During workup, to conserve labeled compound, the fractions from the AG1-X8 column that contained a mixture of PEP and 2-PGA were combined and rechromatographed on AG1-X8. After the second column, any fractions still containing labeled PEP were resubmitted to incubation with enolase. The potassium salt of the [3,3-$^2H_2$]-2-PGA was stored as a freeze-dried powder at −78 °C. A ^{1}H NMR analysis of the labeled 2-PGA showed that 70–75% of the protons on C-3 were deuterons, confirming the loss of label in the conversion of pyruvate into PEP. Subsequent mass spectral integrations indicated that 70.0 ± 0.5% of the protons on C-3 were deuterons. ^{1}H NMR (400 MHz, D_2O): δ 4.02 (0.22H, dd), 4.11 (0.22H, dd), 4.66 (1H, dd).

Secondary Deuterium Isotope Effect by Intermolecular Competition. The secondary deuterium isotope effect for the enolase-catalyzed reaction was determined using the same procedure as described for the primary deuterium isotope effect. A mixture of 2-PGA (0.817 mM), [3-^{2}H]-2-PGA (0.294 mM), and [3,3-$^2H_2$]-2-PGA (0.376 mM) was preincubated at 25 °C in 50 mM potassium MES buffer (15 mL), pH 6.55, containing ADP (1.86 mM), NADH (2.31 mM), $MgSO_4$ (0.5 mM), glucose (1.34 mM), hexokinase (20 units), lactate dehydrogenase (120 units), and pyruvate kinase (200 units). Enolase (2–4 units) was added, and samples were taken after 40% and 60% overall reaction. The samples were processed for mass spectral analysis as described.

Synthesis of 2,3,3-Trideuterio-2-phosphoglycerate. 2,3,3-Trideuterio-2-phosphoglycerate ([2,3,3-$^2H_3$]-2-PGA) was prepared enzymatically from 3,3-dideuteriophosphoenolpyruvate ([3,3-$^2H_2$]PEP) using the same procedure as that described above for the synthesis of [3,3-$^2H_2$]-2-PGA, only the conversion was conducted in buffered D_2O.

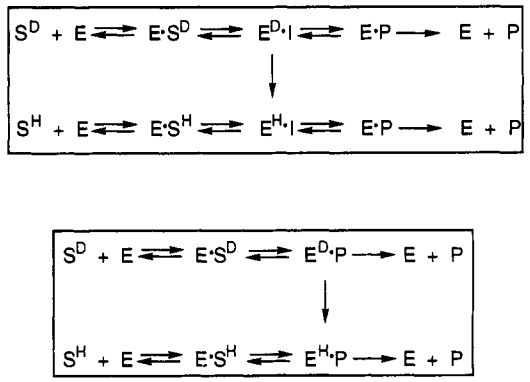
Double Isotope Fractionation Experiment. The double isotope fractionation experiments were conducted using the procedure described for the secondary deuterium isotope effects. A mixture of [2-^{2}H]-2-PGA (0.897 mM), [2,3,3-$^2H_3$]-2-PGA (0.274 mM), and [2,3-$^2H_2$]-2-PGA (0.317 mM) was incubated at 25 °C in 50 mM potassium MES buffer (15 mL), pH 6.55, containing ADP (1.86 mM), NADH (2.31 mM), $MgSO_4$ (0.5 mM), glucose (1.34 mM), hexokinase (20 units), lactate dehydrogenase (120 units), and pyruvate kinase (200 units). Enolase (2–4 units) was added, and samples were taken after approximately 40% and 60% overall reaction. The 2-PGA-containing HPLC fractions were combined, and the samples were processed for mass spectral analysis as before.

THEORY

Determination of Kinetic Isotope Effects by Intermolecular Competition. Intermolecular competition experiments determine the relative rate constants for the reaction of two or more isotopically-labeled substrates undergoing the same irreversible process. Such ratios of rate constants can also be obtained from direct kinetic measurements of the labeled and unlabeled substrates separately, but the values obtained from competitive experiments are, in general, more accurate. The competitive approach guarantees that both substrates experience *exactly* the same reaction conditions and avoids apparent differences in rate that can be caused by slight variations in extrinsic variables, such as pH, ionic strength, or enzyme activity. In a competitive experiment, a mixture of labeled and unlabeled substrate is prepared, and the conversion of substrate to product is initiated by the addition of enzyme. If the reaction exhibits a normal primary isotope effect, the concentration of labeled substrate relative to that of unlabeled substrate will increase over time.

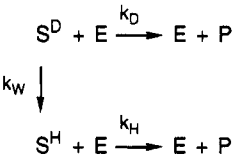
To determine the different kinetic isotope effects for the enolase-catalyzed reaction using data obtained from competitive experiments, the relationship between the measured isotopic enrichment at various extents of reaction and the kinetic isotope effect must be examined. The enolase-catalyzed reaction is complicated by the "washout" of isotopic label due to the enzyme-catalyzed exchange of the C-2 proton of 2-PGA with solvent water, and the standard analysis used for heavy isotope effect measurements is, therefore, not applicable. The desired relationship depends on the assumed mechanistic scheme for the reaction, separate schemes being required for the stepwise and concerted mechanisms. These schemes are presented in Scheme 1a,b, respectively, and were chosen to include the minimum number of different enzyme forms for the particular mechanistic route.

Scheme 1: Mechanistic Schemes for the Enolase-Catalyzed Reaction Assuming a Stepwise Mechanism (A) and a Concerted Mechanism (B)[a]



[a] Abbreviations: S^D, deuterio-2-PGA; S^H, protio-2-PGA; I, a carbanionic intermediate; P, PEP; E, enolase.

Scheme 2: Mechanistic Scheme for the Enolase-Catalyzed Reaction in Terms of Net Rate Constants[a,b]



[a] Abbreviations: S^D, deuterio-2-PGA; S^H, protio-2-PGA; P, PEP; E, enolase. [b] k_H and k_W have been defined somewhat differently here than in eqs 4 and 5. The rate constants described here are more general, taking into account many possible mechanistic approaches including the specific case under consideration in eqs 4 and 5.

The full expression for the level of isotopic enrichment of all species at various extents of conversion as a function of the many kinetic rate constants in these schemes would be complex. But since the competition experiments have been analyzed by determining the isotopic composition of the remaining substrate after various extents of reaction, the only events that are followed are the disappearance of 2-PGA (*i.e.*, the conversion of substrate to product) and changes in the isotopic composition of the remaining 2-PGA. We therefore define three net rate constants, representing the conversion of deuterio-2-PGA to product (k_D), the conversion of protio-2-PGA to product (k_H), and the conversion of deuterio-2-PGA to protio-2-PGA (k_W).

Concentrating on the overall conversions represented by the three net rate constants, the two mechanistic schemes can be simplified and condensed into a single formulation (Scheme 2). The results obtained from the competition experiments were analyzed using this formulation.

Primary ^2H, Primary ^{18}O, and Secondary ^2H Isotope Effects. Competition experiments were used in the determination of the primary deuterium effect, the primary ^{18}O effect, and the secondary deuterium kinetic isotope effect for the enolase-catalyzed reaction. The data obtained from the competition experiments were analyzed using eq 1, which relates to the kinetic scheme shown in Scheme 2.

$$H = (D^{k_H/k_{DW}})\left[\left(\frac{k_W}{k_H - k_{DW}}\right)(D^{(-k_H+k_{DW})/k_{DW}} - D_0^{(-k_H+k_{DW})/k_{DW}}) + (H_0 D_0^{-k_H/k_{DW}})\right] \quad (1)$$

where $k_{DW} = k_D + k_W$, H = concentration of unlabeled

substrate after partial reaction (S^H), H_0 = concentration of unlabeled substrate at time zero, D = concentration of deuterated substrate after partial reaction (S^D), and D_0 = concentration of deuterated substrate at time zero.

In contrast to the primary deuterium experiments, the primary ^{18}O and secondary deuterium experiments will exhibit no significant washout of the ^{18}O label or of the deuterium labels at C-3 of 2-PGA. The absence of washout allows eq 1 to be simplified to eq 2. This equation can then be re-expressed

$$H = H_0(D/D_0)^{k_H/k_D} \quad (2)$$

in the more useful form of eq 3, in which the value of the kinetic isotope effect (α) is a direct function of the extent of reaction and the isotope peak ratios. The results obtained from the primary ^{18}O experiments and the secondary deuterium competition experiments were analyzed using eq 3.

$$\alpha = \frac{\log [c(1 + R_0)/c_0(1 + R)]}{\log [cR(1 + R_0)/c_0R_0(1 + R)]} \quad (3)$$

where α = kinetic isotope effect, c = concentration of substrate after partial reaction, c_o = concentration of substrate at time zero, R = ratio of labeled and unlabeled substrate after partial reaction, and R_o = ratio of labeled and unlabeled substrate at time zero.

The calculation of the secondary deuterium isotope effect is more complex when the C-2 carbon carries a deuterium, because of the existence of concomitant washout of the isotope from this position. A simple comparison of the M + 1, M + 2, and M + 3 mass peaks no longer provides enough information to obtain the kinetic isotope effect. The problem derives from the fact that isotopic washout from the C-2 position increases the number of variously labeled substrate species as the reaction proceeds. Prior to the addition of enzyme, the substrate mixture is comprised of the three C-2 deuterated species: [2-$^2H_1$]-2-PGA, [2,3-$^2H_2$]-2-PGA, and [2,3,3-$^2H_3$]-2-PGA. After the addition of enzyme, three additional substrate species are formed due to the exchange of the C-2 deuteron with solvent H_2O: unlabeled-2-PGA, [3-$^2H_1$]-2-PGA, and [3,3-$^2H_2$]-2-PGA. The resulting six substrates are, moreover, degenerate when analyzed by mass spectroscopy, corresponding to only four distinct mass peaks.

In the double isotope fractionation experiments, the secondary C-3 deuterium isotope effects are compared for 2-PGA that carries either a proton or a deuteron at C-2. Unfortunately, exchange of the C-2 deuteron for a solvent proton, the primary deuterium isotope effect, and the desired secondary deuterium isotope effect all concurrently affect the production of PEP when the 2-PGA is labeled with deuterium at both the C-2 and C-3 carbons. Changes in the substrate concentrations at various extents of reaction are too complicated to be interpreted directly, and the complexity is further compounded by the degeneracy of the associated mass peaks. To determine the amount of enrichment in C-3 deuterio-2-PGA relative to the C-3 protio-2-PGA, therefore, it is necessary to first "correct" the observed mass peak ratios for changes due to washout and for the primary deuterium isotope effect. The "corrected ratios" can then be used to calculate the secondary deuterium kinetic isotope effect for the C-2 deuterated substrate using eq 3, as was done for the C-2 unlabeled substrate.

Intrinsic Secondary Deuterium Isotope Effect for the Concerted Mechanism. If the enolase-catalyzed reaction is concerted, the intrinsic secondary deuterium isotope effect associated with OH^- removal can be estimated based on the experimental measurement of k_W/k_H and the secondary deuterium equilibrium isotope effect. For this mechanism, if one assumes that the exchange of the abstracted proton with solvent water is relatively rapid, the ratio k_W/k_H represents the partitioning of the enolase–PEP complex, as depicted in Figure 1a. Then

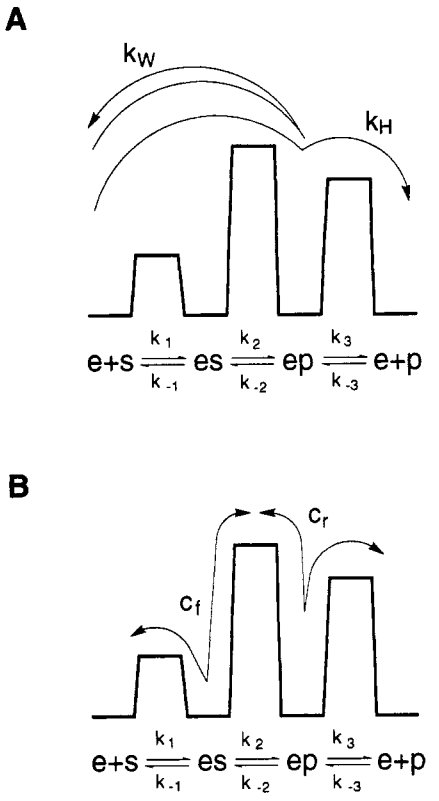


FIGURE 1: Definition of the intrinsic secondary deuterium isotope effect for the concerted mechanism. (A) k_W/k_H, the partioning of the enolase–PEP complex backwards and forwards. (B) Showing the forward and reverse commitments for the isotopically-sensitive step.

$$\frac{k_W}{k_H} = \frac{k_{-1}k_{-2}/(k_{-1} + k_2)}{k_3} \quad (4)$$

If the forward and reverse commitments (c_f and c_r, Figure 1b) are defined as k_2/k_{-1} and k_{-2}/k_3, respectively, eq 4 can be expressed as

$$\frac{k_W}{k_H} = \frac{c_r}{1 + c_f} \quad (5)$$

Further, the equation relating an observed kinetic isotope effect to the intrinsic isotope effect is given by

$$\alpha_{obs} = \frac{\alpha_{int} + c_f + c_r({}^{D_2}K_{eq})}{1 + c_f + c_r} \quad (6)$$

where α_{obs} is the observed secondary deuterium isotope effect, α_{int} is the intrinsic secondary deuterium isotope effect, and ${}^{D_2}K_{eq}$ is the corresponding equilibrium isotope effect. This expression can then be rearranged to eq 7, which defines the intrinsic isotope effect as a function of the partitioning of ep (Figure 1a) and the measured secondary equilibrium and kinetic isotope effects.

$$\frac{\alpha_{int} + c_f}{1 + c_f} = \alpha_{obs} + \frac{k_W}{k_H}[\alpha_{obs} - {}^{D_2}K_{eq}] \quad (7)$$

Table 1: Primary Deuterium Equilibrium Isotope Effect

pH	buffer	K_{eq}^{H}	K_{eq}^{D}	$(K_{eq}^{H}/K_{eq}^{D})^{a}$
5.55	MES	1.97	1.65	1.26
6.65	MES	3.47	3.00	1.23
7.70	MOPS	4.75	4.06	1.24

[a] A correction of 0.945 was used when calculating the equilibrium isotope effects from K_{eq}^{H} and K_{eq}^{D} to account for differences in the fractionation factors of HOD and D_2O (Cleland, 1980).

This analysis is, however, applicable only to the concerted mechanism where exchange can occur only following the loss of OH^-. For the stepwise mechanism, washout can occur either before or after the rehybridization at C-3. If exchange of the C-2 proton occurs before the loss of OH^-, the relative rate of washout will be a function of the forward and reverse commitments for the transition associated with H^+ removal but not that for OH^- loss and will, therefore, no longer limit the size of the intrinsic secondary deuterium isotope effect associated with the rehybridization at C-3. Such an analysis is thus not useful for a stepwise reaction.

RESULTS

Primary Deuterium Equilibrium Isotope Effects. The primary deuterium equilibrium isotope effect for the enolase-catalyzed reaction was obtained from the individual equilibrium constants, $^{H}K_{eq}$ and $^{D}K_{eq}$, at various pH values (Table 1). The equilibrium isotope effect ($^{H}K_{eq}/^{D}K_{eq}$) was found experimentally to be 1.24. This value is consistent both with the values of 1.13–1.18 predicted by the calculations of Hartshorn and Shiner (1972) and with the experimental values of 1.19 and 1.25 reported by Cleland (1980) and Anderson (1981), respectively.

Primary Deuterium Kinetic Isotope Effect by Intermolecular Competition. To avoid problems that could arise in the determination of the primary deuterium and/or primary ^{18}O kinetic isotope effects due to the concomitant solvent isotope effects present when the reaction is observed in the hydration direction, all of the studies reported here have been conducted as dehydration reactions. The kinetic isotope effect associated with deuterium substitution at C-2 of 2-PGA was determined competitively. The conversion of 2-PGA to PEP was made irreversible by converting the PEP formed during the reaction to lactate using pyruvate kinase and lactate dehydrogenase. The equilibrium constants for pyruvate kinase and lactate dehydrogenase at pH 7.4 are approximately 10^4 and 10^6, respectively, making the overall reaction from 2-PGA to lactate effectively irreversible. Hexokinase and glucose were included as part of the coupled system to minimize changes in the concentration of Mg(II) over the course of the reaction. Both ADP and ATP chelate Mg(II), but the affinity of ATP for Mg(II) is approximately 10-fold higher than that of ADP, and a buildup of ATP over the course of the reaction would decrease the effective Mg(II) concentration. This, in turn, would increase the magnitude of the enolase kinetic isotope effect. Hexokinase effectively "buffers" the concentration of Mg(II) by returning the ATP that is formed during the reaction back to ADP.

The primary deuterium competition experiments were conducted by adding enolase to a mixture of [2-^{1}H]-2-PGA and [2-^{2}H]-2-PGA. Portions of the reaction mixture were removed at various times, and the reisolated 2-PGA was partially purified by HPLC. These samples were then derivatized and submitted to mass spectral analysis to determine the isotopic composition of the 2-PGA.

Table 2: Mass Peak Integrations for the Primary Deuterium Competition Experiment

	extent of reaction (%)	isotopic ratio:[a] (M + 1)/M = 190/189 (×100)	standard deviation
run 1[b]	0.0	48.67	0.13
	40.9	61.78	0.08
	61.6	72.40	0.05
run 2	0.0	48.69	0.03
	40.4	61.14	0.04
	59.0	71.54	0.05

[a] The isotope ratio peaks have been corrected for the natural abundance of the M + 1 mass peak. The substrate peak (M) was normalized to 100, and a value of 8.058 was used as the "observed" natural abundance of M + 1. [b] Runs 1 and 2 are duplicate runs. The two sets of data were obtained from separate competitive experiments.

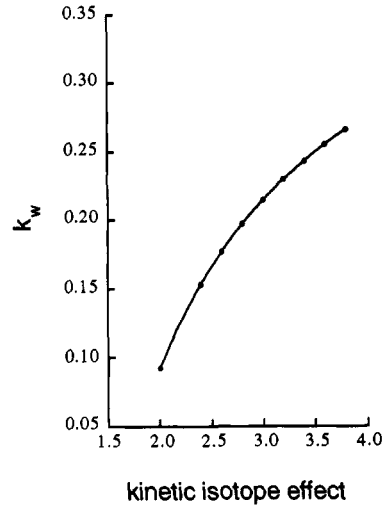


FIGURE 2: Relationship between the primary deuterium kinetic isotope effect and the rate of isotopic washout (k_w) for the competition experiments.

The labeled pentafluorobenzyl glycerate samples were analyzed by direct ratio mass spectroscopy according to the method of Kirsch and Rosenberg (1979). The resulting mass spectrum showed peaks at molecular weights of 189, 190, and 191, corresponding to the M, M + 1, and M + 2 isotope peaks, respectively. The spectrum showed no significant ion fragmentation. A gas chromatogram was obtained, and the resulting peaks were integrated numerically. The resulting mass peak ratios for the samples at various extents of reaction are shown in Table 2.

An attempt was made to calculate the kinetic isotope effect and the "washout" rate from the observed mass peak integrations using eq 1. The concentrations of [2-^{1}H]-2-PGA and [2-^{2}H]-2-PGA present after partial conversion to product were calculated from the initial concentration of 2-PGA, the extent of reaction, and the corresponding mass peak ratios. The concentration of [2-^{1}H]-2-PGA ("H" in eq 1) was plotted against the concentration of [2-^{2}H]-2-PGA ("D" in eq 1), and an attempt was made to fit the data. To derive the two rate constants ratios, k_D/k_H and k_W/k_H, k_H was set to 1.0. We found that rather than providing single values of k_D/k_H and k_W/k_H, the curve fitting routine generated a *set* of k_D, k_W combinations that closely fitted the observed results. This finding is illustrated in Figure 2.

The relationship shown in Figure 2 arises because a set of combinations of values of the kinetic isotope effect and of k_W results in concentrations of [2-^{2}H]-2-PGA and [2-^{1}H]-2-PGA that agree with those obtained experimentally. If one starts

Table 3: Mass Peak Integrations for the Deuterium Washout Experiment

	extent of reaction (%)	isotopic ratio:[a] M/(M + 1) = 189/190 (×100)	standard deviation
run 1[b]	0.0	1.24	0.03
	3.4	3.90	0.04
	9.4	7.19	0.02
run 2	0.0	1.30	0.01
	8.1	6.92	0.01
	12.2	9.18	0.01

[a] The isotope ratio peaks have been corrected for the natural abundance of the M + 1 mass peak. The substrate peak (M) was normalized to 100, and a value of 8.058 was used as the "observed" natural abundance of M + 1. [b] Runs 1 and 2 are duplicate runs. The two sets of data were obtained from separate competitive experiments.

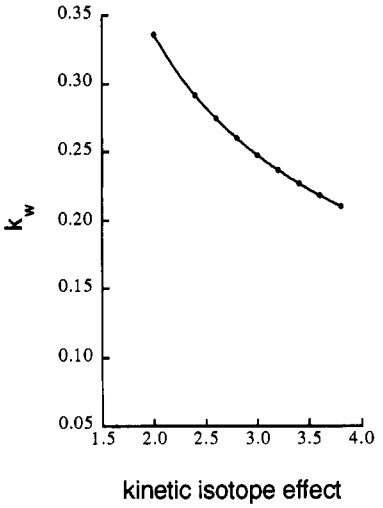


FIGURE 3: Relationship between the primary deuterium kinetic isotope effect and the rate of isotopic washout (k_w) for the deuterium washout experiments.

with a mixture of labeled and unlabeled substrate, increasing the kinetic isotope effect will obviously raise the ratio of remaining [2-^{2}H]-2-PGA to remaining [2-^{1}H]-2-PGA. This rise can be "overcome" by increasing k_W, which converts [2-^{2}H]-2-PGA into [2-^{1}H]-2-PGA. A given set of observed concentrations of [2-^{2}H]-2-PGA and [2-^{1}H]-2-PGA can therefore be accurately modeled by an appropriate pair of values of the kinetic isotope effect and k_W. The interdependence of these parameters is expressed in Figure 2.

Determination of the Washout Rate for the C-2 Proton. Since unique values for k_D/k_H and k_W cannot be determined from the competition data alone, the rate of enolase-catalyzed isotopic washout from C-2 of 2-PGA (k_W) was determined independently. Enolase was added to a solution of [2-^{2}H]-2-PGA in MES buffer at pH 6.65. Samples were taken at times during the first 12% of reaction, before the buildup of [2-^{1}H]-2-PGA was significant. These samples were processed as before, and the mass peak ratios obtained are presented in Table 3.

The concentrations of [2-^{1}H]-2-PGA and [2-^{2}H]-2-PGA were calculated from the initial concentration of 2-PGA, the extent of reaction, and the corresponding mass peak ratios. The concentration of [2-^{1}H]-2-PGA was plotted against the concentration of [2-^{2}H]-2-PGA, and these data were fitted to eq 1 as for the competition experiments. A second set of k_D, k_W combinations was obtained, and this is presented in Figure 3.

At first sight, it may seem odd that the trend observed for the washout data (Figure 3 and Figure 4, dashed line) is opposite to that obtained for the competition data (Figure 2 and Figure 4, solid line), since both experiments are interpreted using the same mechanistic scheme. However, these relationships simply reflect the fact that the two sets of experiments are conducted differently. The reason behind the slope of the curve in Figure 2 has been explained above. For the washout experiments (Figure 3 and Figure 4, dashed line), we start with one substrate, C-2 deuterio-2-PGA, which reacts to form product either directly or by first being converted (by washout) to unlabeled 2-PGA (Scheme 2). If the isotope effect were to rise (i.e., k_D becomes smaller than k_H), a higher proportion of substrate would follow the route via unlabeled 2-PGA. Further, if k_W were to fall, a *smaller* proportion of substrate would proceed through unlabeled 2-PGA. For the washout experiment, therefore, the experimental data can be modeled by sets of values of k_w and the kinetic isotope effect, where a higher isotope effect compensates for a lower k_w. This explains the nature of the relationship illustrated in Figure 3.

Determination of the Primary Deuterium Isotope Effect and Rate of Washout. Both the competition and the washout experiments have as their foundation the same overall reaction, the enolase-catalyzed conversion of 2-PGA to PEP, and the kinetic isotope effect must have a unique value. Since for the competition experiments an *in*crease in the kinetic isotope effect corresponds to an increase in the value of k_W, and for the washout experiments an increase in the kinetic isotope effect corresponds to a *de*crease in the value of k_W, unique values for the kinetic isotope effect and k_W can be found by plotting these two curves simultaneously (Figure 4). From the region of intersection of the curves, we can conclude that the primary deuterium kinetic isotope effect for the enolase-catalyzed reaction at pH 6.55 is 3.26 ± 0.12 and that the rate of enzyme-catalyzed "washout" (k_W) is 23.4 ± 0.6% the rate of turnover (k_H).[2]

Primary ^{18}O Isotope Effect by Intermolecular Competition. Measurement of the primary ^{18}O isotope effect for the enolase-catalyzed reaction was attempted using an intermolecular competition experiment, as for the primary deuterium experiments. Enolase was added to a mixture of unlabeled 2-PGA and [3-^{18}O]-2-PGA. Samples were removed over the course of the reaction, and the remaining 2-PGA was analyzed by mass spectroscopy (Table 4).

The presence of a "normal" ^{18}O isotope effect would cause the ^{18}O/^{16}O ratio of reisolated substrate to increase during the reaction. From Table 4, however, the observed ratios evidently *de*crease. This trend suggests that the enzyme also catalyzes washout of the ^{18}O label from C-3. Such washout could reflect the fact that OH^- removal is somewhat faster than the rate of turnover, i.e., that the release of OH^- from the enzyme is faster than product release. Indeed, Dinovo and Boyer (1971) have reported the existence of this exchange reaction. The decrease could, however, also derive from the enolase-catalyzed rehydration of product PEP. Although excess pyruvate kinase was included in the competition experiments to trap PEP as it is formed, any inadequacy in this trapping would result in the production of unlabeled 2-PGA. Whatever the reason, no conclusion can be reached

[2] The primary deuterium isotope effect reported here is consistent with that obtained previously using the technique of equilibrium perturbation. Using this approach, Anderson (1981) found primary deuterium isotope effects of 3.5 at pH 5.5, 2.4 at pH 6.9, 2.0 at pH 7.7, and 1.5 at pH 8.32.

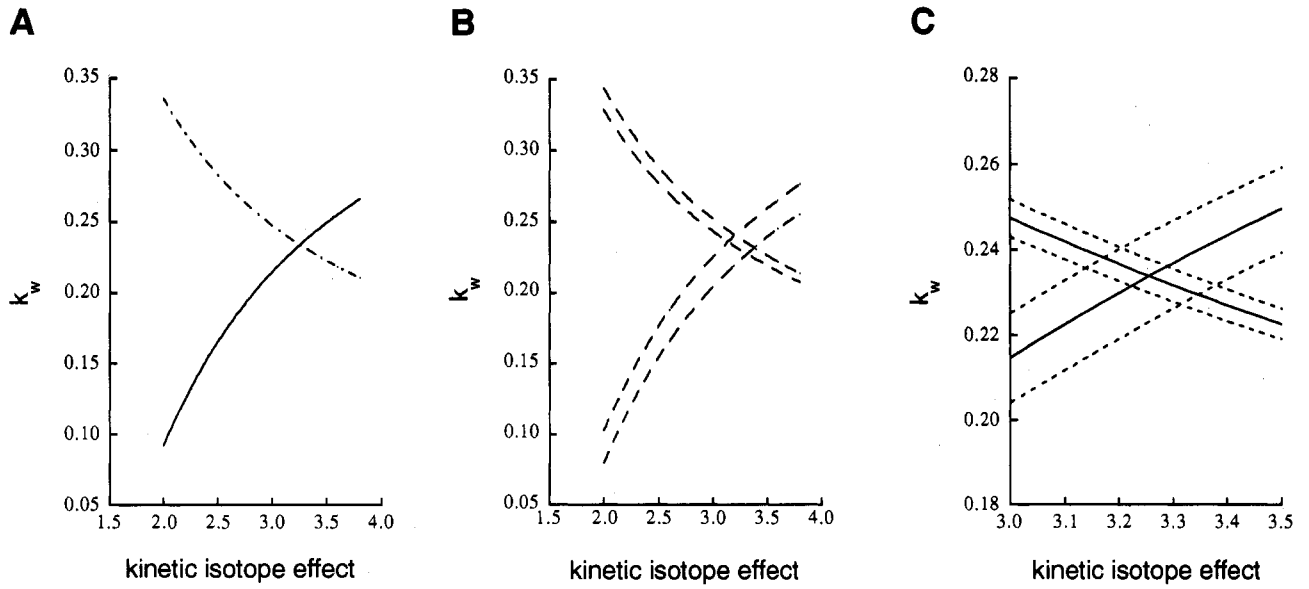


FIGURE 4.

Table 4: Mass Peak Ratios for the Primary ^{18}O Competition Experiments

	extent of reaction (%)	isotopic ratio:[a] (M + 2)/M = 191/189 (×100)	standard deviation
run 1[b]	0.0	29.45	0.05
	31.0	28.64	0.06
	56.5	26.69	0.09
run 2	0.0	29.45	0.05
	33.7	28.65	0.06
	55.1	27.21	0.07
run 3	0.0	30.09	0.02
	26.0	29.77	0.09
	44.8	29.60	0.02
run 4	0.0	30.09	0.02
	25.4	28.95	0.05
	42.3	29.03	0.10

[a] The isotope ratio peaks have been corrected for the natural abundance of the M + 2 mass peak. The substrate peak (M) was normalized to 100, and a value of 1.574 was used as the "observed" natural abundance of M + 2. [b] Runs 1–4 are separate competitive experiments. However, runs 1 and 2 and runs 3 and 4 were conducted using the same stock solution.

Table 5: Mass Peak Ratios for the Secondary Deuterium Competition Experiments

		[3-$^2H_1$]-2-PGA		[3-$^2H_2$]-2-PGA	
	extent reaction (%)	isotopic ratio:[a] (M + 1)/M = 190/189 (×100)	standard deviation	isotopic ratio:[a] (M + 2)/M = 191/189 (×100)	standard deviation
run 1	0.0	37.27	0.11	46.63	0.24
	32.2	37.26	0.11	48.44	0.16
	56.2	38.58	0.05	50.92	0.04
run 2	0.0	36.26	0.03	46.99	0.08
	30.5	37.10	0.04	48.48	0.12
	50.5	38.40	0.05	49.75	0.10
run 3	0.0	58.29	0.13	73.66	0.15
	35.7	60.57	0.11	78.54	0.21
	60.9	62.59	0.15	82.48	0.03
run 4	0.0	36.60	0.05	46.06	0.08
	34.5	37.55	0.06	47.75	0.06
	63.6	39.19	0.09	50.67	0.06

[a] The isotope ratio peaks have been corrected for the natural abundance of the M + 1 and M + 2 mass peaks. The substrate peak (M) was normalized to 100. Values of 8.058 and 1.574 were used as the "observed" natural abundance of the M + 1 and M + 2 peaks, respectively.

from these experiments about the presence of an ^{18}O kinetic isotope effect.[3] To probe the timing of the cleavage of the carbon–oxygen bond, therefore, changes at C-3 were investigated by determining the secondary deuterium isotope effect at this center.

C-3 Secondary Deuterium Isotope Effects. The secondary deuterium competition experiments were conducted by adding enolase to a mixture of unlabeled 2-PGA, [3-$^2H_1$]-2-PGA, and [3,3-$^2H_2$]-2-PGA at pH 6.65. Samples were removed over the course of the reaction, and the remaining 2-PGA was analyzed by mass spectroscopy. The mass peak ratios are given in Table 5. These data result in secondary isotope effects of 1.076 ± 0.034 and 1.111 ± 0.038 for the singly and doubly-labeled substrates, respectively.[4]

Intrinsic Secondary Deuterium Isotope Effect for a Concerted Reaction. Having determined the relative rate of washout for the C-2 proton and the secondary deuterium kinetic isotope effect for [3,3-$^2H_2$]-2-PGA, an estimation of the intrinsic secondary deuterium isotope effect can be made for the concerted mechanism. For this mechanism, the relative rate of washout of 0.23 provides a lower limit for the quantity $c_r/(1 + c_f)$ from eq 4. This relationship is exact if proton exchange with solvent water is rapid, whereas, if the proton is "sticky" the measured amount of deuterium in unconverted substrate will be reduced, and consequently $c_r/(1 + c_f) > 0.23$. Given an equilibrium isotope effect for the doubly-deuterated substrate of 1.64 (Anderson, 1981), the intrinsic isotope effect can be expressed as a function of c_f:

[3] Although we were unable to measure a primary ^{18}O isotope effect because of the apparent washout of ^{18}O from the C-3 position of 2-PGA, primary ^{18}O isotope effects of 1.03 at pH 5.5, 1.02 at pH 7.1, and 1.0 at pH 9.1 have been observed using the technique of equilibrium perturbation (Anderson, 1981).

[4] The secondary deuterium isotope effects reported here are again consistent with the effects determined previously using the technique of equilibrium perturbation. Using this approach, Anderson (1981) found secondary deuterium isotope effects for doubly-labeled 2-PGA of 1.16 at pH 5.5, 1.13 at pH 7.2, and 1.04 at pH 9.1.

$$\frac{\alpha_{int} + c_f}{1 + c_f} = 1.11 + 0.23(1.11 - 1.64) = 0.99 \quad (8)$$

or

$$\alpha_{int} = 0.99 + (0.99 - 1)c_f \quad (9)$$

On the basis of this relationship, it is apparent that the intrinsic secondary deuterium isotope effect is very near unity. The value of c_f would have to be quite large to alter the value of this effect away from unity. This is quite improbable, since the observed primary deuterium isotope effect of 3.2 limits the size of the forward commitment. A c_f of 2.0 already corresponds to an intrinsic primary deuterium effect of over 7.

These calculations show that, for a concerted reaction, the intrinsic isotope effect does not differ significantly from unity. If our assumption that exchange of the removed proton is rapid were wrong, eq 9 would require an intrinsic isotope effect that is even smaller, that is, less than unity. An inverse intrinsic secondary effect is neither anticipated nor precedented for a dehydration reaction, and we may conclude that slow exchange of the removed proton for the concerted mechanism is very improbable.

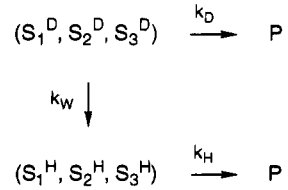
Double Isotope Fractionation Test. The secondary deuterium isotope effect for the enolase-catalyzed reaction when the C-2 carbon of 2-PGA carries a deuterium was also determined. In preparing the substrates for the double isotope fractionation experiments, the C-3 deuterated PEP was the same as that used in the preparation of the substrates for the secondary deuterium isotope effect experiments. This is crucial, since, to compare the values obtained for the secondary deuterium isotope effect when C-2 carries a deuterium to that when this position is unlabeled, the extent and distribution of the label at C-3 must be the same.

The double deuterium fractionation experiments were conducted by adding enolase to a mixture of [2-^{2}H]-2-PGA, [2,3-$^2H_2$]-2-PGA, and [2,3,3-$^2H_3$]-2-PGA at pH 6.65. Samples were removed during the reaction, and the remaining 2-PGA was analyzed by mass spectroscopy.

As discussed above, if there were no washout of the isotope from C-2, the calculation of the secondary isotope effect when C-2 is deuterated would be identical to that for the secondary deuterium isotope effect when this position is unlabeled. We have seen, however, that enzyme-catalyzed washout occurs during the conversion from 2-PGA to PEP. The calculation of the secondary isotope effect for C-2 deuterated substrate becomes much more complex with the inclusion of washout. As discussed earlier, this complexity derives from the enolase-catalyzed exchange of the C-2 deuteron with solvent H_2O, such exchange increasing the number of substrate molecules from three to six as the reaction proceeds. The resulting six substrate species, moreover, correspond to only four (degenerate) mass peaks, and, thus, the relative concentrations of the six labeled species can no longer be determined directly from the integration of the mass peaks. To determine the amount of enrichment in C-3 deuterio-2-PGA relative to the C-3 protio-2-PGA, it was necessary to correct the observed mass peak ratios for changes due to the washout of deuterium from the C-2 position and for the primary deuterium isotope effect.

The primary deuterium isotope effect and the relative rate of washout have been determined to be 3.26 ± 0.12 and 23.4 ± 0.6%, respectively. Using these values in conjunction with the initial substrate concentrations for each of the species, we can predict the concentrations of each of the various substrates over the course of the reaction, assuming the presence of *only* a primary deuterium isotope effect and enzyme-catalyzed washout. The calculated concentrations of the six substrates can then be used to derive the secondary deuterium isotope effect for C-2 deuterated substrate.

Scheme 3: Mechanistic Scheme Used for the Simulation of the Double Isotope Fractionation Experiments[a]



[a] Abbreviations: S_1^D, [2-$^2H_1$]-2-PGA; S_2^D, [2,3-$^2H_2$]-2-PGA; S_3^D, [2,3,3-$^2H_3$]-2-PGA; S_1^H, 2-PGA; S_2^H, [3-$^2H_1$]-2-PGA; S_3^H, [3,3-$^2H_2$]-2-PGA; P, PEP.

Table 6: Mass Peak Ratios for the Double Isotope Fractionation Experiments[a]

extent of reaction (%)	M + 2 peak integration[b] (191)		M + 3 peak integration[b] (192)	
	calcd	obsd	calcd	obsd
0.0	30.68	30.68	35.45	35.45
30.2	34.38	35.05	33.62	34.71
30.3	34.39	35.08	33.62	34.71
30.8	34.44	35.05	33.60	34.71
31.0	34.46	35.08	33.59	34.71
49.8	36.33	37.05	32.84	34.91
50.0	36.35	38.06	32.83	35.30
50.8	36.42	37.05	32.81	34.91
51.0	36.43	38.06	32.80	35.30

[a] These data are from one of many duplicate runs conducted to determine the secondary deuterium isotope effect. [b] Relative to M + 1 = 100.

The calculated concentrations of the six substrate molecules at various extents of reaction were obtained using the modeling program, KINSIM. The simulation program requires a kinetic scheme, the initial substrate concentrations, and values for the kinetic rate constants. The simplified scheme presented in Scheme 3 was used. (In this mechanistic scheme we have ignored the enzyme terms that are present in the earlier scheme (Scheme 2), since the inclusion of enzyme will affect the absolute rate of reaction but not the isotopic composition of 2-PGA for any given extent of reaction.) Initial substrate concentrations were entered into the KINSIM program, along with values for k_H, k_D, and k_W of 1.0, 0.307, and 0.234, respectively. The simulation was then asked to generate the concentrations of the six substrates as a function of the extent of the reaction.

The substrate concentrations generated by KINSIM were used to calculate the expected mass peak ratios at various extents of reaction assuming *only* the primary deuterium isotope effect and washout of the C-2 deuteron (Table 6). To obtain the secondary deuterium isotope effect from these data, the calculated integrations for the M + 2 and M + 3 peaks were subtracted from the corresponding observed values. The resulting differences reflect the additional contribution of the secondary deuterium isotope effect. The secondary deuterium isotope effect for the conversion of C-2 deuterated 2-PGA to PEP was found to be 1.055 ± 0.032 and 1.077 ± 0.034 for the C-3 mono- and dideuterated substrates, respectively.

The C-3 secondary deuterium isotope effects appear to be somewhat smaller when C-2 is deuterated than when this position carries a proton. Yet, due to the relatively large error limits associated with both sets of values, these results are suggestive but not definitive.

DISCUSSION

Primary Deuterium Isotope Effect and Enzyme-Catalyzed Exchange of the C-2 Proton. The primary deuterium isotope effect for the enolase-catalyzed reaction has been determined using intermolecular competition experiments and direct ratio mass spectroscopy. The enolase-catalyzed reaction was found to exhibit a primary deuterium isotope effect of 3.26 ± 0.12 at pH 6.65 and a Mg(II) concentration of 500 μM. Enolase has also been shown to catalyze the relatively rapid exchange of the proton from the C-2 carbon of 2-PGA with solvent water. Such enzyme-catalyzed exchange of the C-2 proton was reported earlier by Dinovo and Boyer (1971). These previous studies were, however, conducted at very high Mg(II) concentrations (25 mM), and since the exchange rate is sensitive to the Mg(II) concentration, the reported rates are not directly applicable to our studies. At pH 7.4 and a Mg(II) concentration of 1 mM, Shen and Westhead (1973) report that no more than 5% of the remaining 2-PGA had exchanged its C-2 proton for a solvent deuteron after 50% conversion to product. We have found a significantly higher rate of exchange; 23% of the 2-PGA remaining after 50% conversion has exchanged its C-2 deuteron for a solvent proton at pH 6.7. This difference may at least partially reflect the presence of a solvent isotope effect, since in the earlier experiments the heavy isotope washes in from the solvent, whereas, in the present work, the heavy isotope washes out into the solvent.

The fact that the enolase-catalyzed reaction exhibits both a primary C-2 deuterium kinetic isotope effect and a significant level of enzyme-catalyzed exchange of the C-2 proton shows both that the transition state associated with proton abstraction is partially rate-limiting and that proton abstraction occurs prior to one or more later transition states that are also kinetically significant. The exchange of the C-2 proton is consistent either with a stepwise mechanism or with a concerted mechanism in which the OH^- fragment is sequestered at the active site.

Secondary Deuterium Isotope Effects. Attempts were made to measure the primary ^{18}O kinetic isotope effect for the enolase-catalyzed reaction. These attempts were unsuccessful, so secondary deuterium isotope effects have been used to evaluate the kinetic significance of the step in which OH^- is lost. The secondary deuterium isotope effects were determined using direct intermolecular competition experiments both when the C-2 carbon carries a proton and when this center is deuterated.

A secondary deuterium *kinetic* isotope effect for the enolase-catalyzed reaction derives from a partial change in the hybridization of the C-3 carbon on going from the ground state to the transition state. Since for the dehydration reaction, C-3 of the substrate is sp^3 and of the product, sp^2, if the step associated with the loss of OH^- is kinetically significant, a secondary deuterium isotope effect of greater than 1.0 would be expected. To complicate matters somewhat, even if this step were not kinetically significant, a secondary deuterium isotope effect may still be observed. A secondary deuterium isotope effect is merely the partial expression of the equilibrium isotope effect associated with the overall change in hybridization of the carbon center under scrutiny. Thus, if the loss of OH^- were rapid, but it occurred before a rate-determining transition state, the labeled substrates would preequilibrate causing the *equilibrium* isotope effect to be expressed as an *apparent* kinetic isotope effect, despite the fact that the loss of OH^- was kinetically insignificant.

In this paper, we report the presence of a normal secondary deuterium isotope effect for the enolase-catalyzed reaction. During the dehydration of 2-PGA to PEP, proton abstraction from C-2 either preceeds or is concurrent with the loss of OH^- from C-3. If the elimination reaction were concerted, a significant amount of C–O bond cleavage (and concomitant rehybridization of the sp^3 carbon) would be anticipated. In the fumarase- and crotonase-catalyzed reactions, for example, where there are significant ^{18}O kinetic isotope effects, the secondary deuterium effects are between 1.13 and 1.23 for a single α-secondary hydrogen (Bahnson & Anderson, 1989; Blanchard & Cleland, 1980). For enolase, with two α-secondary hydrogens, an intrinsic secondary kinetic isotope effect of as large as 1.3–1.4 would be anticipated for the concerted mechanism. Our results limit the size of this secondary isotope effect to 1.11, which suggests that if the reaction mechanism is concerted, the transition state shows very little C–O bond cleavage. This conclusion is further supported by the fact that the intrinsic secondary deuterium isotope effect has been shown to be very near unity. Thus, if the enolase-catalyzed reaction is a concerted process, the transition state must be very asymmetric, making the transition state for the concerted mechanism very similar to that anticipated for a stepwise pathway. The imposition of such tight restrictions on the concerted mechanism would seem to suggest that the enolase-catalyzed reaction is unlikely to proceed in this manner.

If, on the other hand, the reaction is stepwise, our results are easily accommodated by a pathway where the transition state for C–H bond cleavage is primarily rate determining and either C–O bond cleavage and/or the release of product also contributes to the rate limitation (in order to account for the observed washout of the C-2 proton). The relatively small secondary ^2H isotope effects and the results from our attempt to determine the ^{18}O isotope effect also support the hypothesis that the overall reaction rate is partially limited by steps occurring after the conclusion of the chemical events.

Mechanistic Conclusions. Since the enolase-catalyzed reaction has been shown to exhibit a significant primary C-2 deuterium isotope effect, a small secondary C-3 deuterium isotope effect, and enzyme-catalyzed exchange of the C-2 proton with solvent water that is comparable to the rate of substrate turnover, the following mechanistic possibilities can be eliminated: (a) any concerted or stepwise mechanism for which a step other than proton abstraction and/or OH^- loss is cleanly rate limiting, (b) a stepwise mechanism in which proton abstraction is cleanly rate limiting, and (c) a stepwise mechanism where OH^- loss dominates the overall reaction rate. The remaining mechanistic possibilities are as follows: (d) a concerted mechanism for which C–O bond cleavage contributes little or nothing to the transition state and for which chemical transformation and product release are partially rate limiting or (e) a stepwise mechanism in which the rate of H^+ abstraction predominates and one or both of the transition states associated with OH^- loss and product release limit the overall reaction rate. Although formally both d and e are feasible, the restrictions put on the concerted mechanism by the intrinsic secondary isotope effect of unity reported here strongly suggest that the enolase-catalyzed reaction is unlikely to proceed in a concerted manner.

On the basis of our studies and those cited previously, we thus conclude that the enolase-catalyzed reaction proceeds via a stepwise mechanism. Finally, we can address whether the loss of OH^- is a kinetically significant process by comparing the secondary C-3 deuterium isotope effect when a proton lies at C-2 to that when this position is deuterated. If deuterium substitution at C-2 does not affect the secondary C-3 deuterium isotope effect, then H^+ abstraction and OH^- loss must occur

in a stepwise manner with OH^- loss being kinetically *in*significant. If, on the other hand, deuterium substitution at C-2 *lowers* the secondary deuterium isotope effect, then H^+ abstraction and OH^- loss must occur in separate steps with both H^+ removal and OH^- loss being kinetically significant. The results of our double isotope fractionation experiments suggest that the secondary deuterium isotope effect decreases slightly upon ^2H substitution at C-2, supporting the hypothesis that both H^+ abstraction from C-2 and the loss of OH^- from C-3 are kinetically significant processes. This possibility is further supported by the fact that an ^{18}O effect in excess of the equilibrium effect was observed using the technique of equilibrium perturbation.

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