Enzymatic Synthesis and Inhibitory Characteristics of Tartronate Semialdehyde Phosphate†

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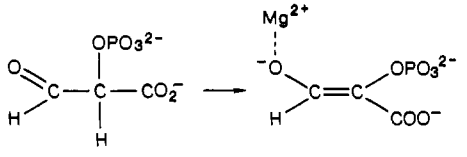
ABSTRACT: The immediate product of the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate is the enol of tartronate semialdehyde phosphate (TSP). The reaction has the same pH profile as that for the phosphorylation of pyruvate with pK's of 8.2 and 9.7 observed in H_2O. This enol tautomerizes in solution to the aldehyde, which in turn becomes hydrated. ^{31}P NMR spectra indicate that the enol resonates ~1 ppm upfield from the hydrated aldehyde. By following the tautomerization spectrophotometrically at 240 nm, we have found it to be independent of pH (0.2 min^{-1} below pH 6 in water), except that it is 2-fold slower above the pK of the phosphate group (6.3 in H_2O and 6.7 in D_2O). It is 3.6-fold slower in D_2O. When this TSP is reduced with $NaBH_4$, ~50% of the product is D-2-phosphoglyceric acid (substrate for enolase). Thus, while the immediate product of the phosphorylation rection is the enol of TSP, the eventual product is D,L-TSP. Both the enol and the aldehyde forms of TSP were found to be potent inhibitors of yeast enolase with apparent K_i values of 100 nM and 5 μM, respectively. However, since the aldehyde form is 95–99% hydrated [Stubbe, J., & Abeles, R. (1980) *Biochemistry 19*, 5505], the true K_i for the aldehyde species is 50–250 nM. The enol of TSP shows slow binding behavior, as expected for an intermediate analogue, with a $t_{1/2}$ for this process of ~15 s (k = 0.046 s^{-1}) and an initial K_i of ~200 nM.

Pyruvate kinase (EC 2.7.1.40) catalyzes the physiological phosphorylation of MgADP by PEP.[1] Other reactions catalyzed by pyruvate kinase include the bicarbonate-dependent phosphorylation of fluoride (Tietz & Ochoa, 1958) and hydroxylamine [as the *N*-hydroxycarbamate (Kupiecki & Coon, 1959; Weiss et al., 1984)], as well as the phosphorylation of glycolate (Kayne, 1974) and other β-substituted carboxylic acids such as D- and L-lactate and D- and L-glycerate (Ash et al., 1984). It has also been observed that β-hydroxypyruvate is phosphorylated to give tartronate semialdehyde phosphate of unknown stereochemistry at C-2 (Weiss & Cleland, 1983; Ash et al., 1984).

The inhibitory characteristics of D-TSP on enolase (EC 4.2.1.11) are rather unique in that D-TSP is acutally a pseudosubstrate or partial substrate. It has been known for some time that enolase catalyzes the enolization of the competitive inhibitor D-TSP (Spring & Wold, 1971b; Lane & Hurst, 1974). The C-2 proton is removed, presumably by some base, to give an enzyme–Mg–enolate complex:



In this study, we have characterized the immediate and eventual products of the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate and determined the inhibitory properties of TSP vs enolase.

Materials and Methods

Materials. Pyruvate kinase and lactate dehydrogenase (both in glycerol) were from Boehringer, as were ATP, DTT, and NADH. NaOD, DCl, and $NaBH_4$ were from Aldrich. D_2O (99.9 atom % D) was from Cambridge Isotope Laboratories. Pyruvate kinase and yeast enolase (both lyophilized), EDTA, pyruvate, β-hydroxypyruvate, PEP, D-2PGA, and iodoacetate were from Sigma.

Assay Procedures. The stability of β-hydroxypyruvate was determined under a number of conditions. The effect of pH, Mg^{2+}, and EDTA was monitored by assaying for residual keto acid with lactate dehydrogenase and NADH at various time intervals of exposure to the particular condition (β-hydroxypyruvate had a V/K value 36% that of pyruvate under identical conditions). The phosphorylation of β-hydroxypyruvate catalyzed by pyruvate kinase was initially followed by performing fixed-time assays for residual keto acid as described above; however, a continuous assay was subsequently used. In this case, the phosphorylation reaction was monitored directly by following the change in absorbance at 240 nm (A_{240}) with a Cary 118 dual-wavelength spectrophotometer equipped with a water bath and thermospacers for accurate temperature control. Because of the intense absorbance due to the adenine moiety of ATP and ADP at 240 nm, the reference cuvette contained the same concentration of adenine (as ATP) as the reaction cuvette (as ATP and ADP). Reaction cuvettes typically contained the following in 3-mL volumes at 25 °C: 50 mM potassium acetate, 1 mM magnesium acetate, 167 μM ATP, 1 mM β-hydroxypyruvate, and 50 mM buffer (Hepes at pH 7 and 7.5, Taps at pH 7.5, 8, and 8.5, Ches at pH 8.5, 9, and 9.5, and Caps at pH 9.5, 10, and 10.5). The same buffers were used in D_2O but at a range 0.5 pH unit higher than that in H_2O due to the equilibrium isotope effect on the acid dissociation constants of the buffers (Schowen, 1977),

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[1] Abbreviations: TSP, tartronate semialdehyde phosphate; PEP, phosphoenolpyruvate; AEP, 3-aminoenolpyruvate 2-phosphate; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Taps, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid; D-2PGA, D-2-phosphoglyceric acid; ppm, parts per million; DTNB, 5,5'-dithiobis(2-nitrobenzoate); DTT, dithiothreitol.

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and the pD was determined by adding 0.4 pH unit to the pH meter reading. Reactions were initiated by the addition of 100 units (for reaction of PEP) of pyruvate kinase in glycerol. It should be noted that TSP reacts with ammonia to produce 3-aminoenolpyruvate 2-phosphate (Spring & Wold, 1971a; Weiss & Cleland, 1983), and thus care was taken to exclude ammonia from these reactions. For reactions carried out in D_2O, all of the above components except pyruvate kinase were lyophilized from D_2O prior to preparation of stock solutions in D_2O. This direct assay method allowed determination of the rate constants for both the initial enzyme-dependent increase and eventual enzyme-independent decrease in A_{240}. Initial velocities for the first portion of the reaction were obtained in D_2O at pD 9.2 and 25 °C by varying the amount of β-hydroxypyruvate at fixed levels of MgATP and monitoring the increase in A_{240}. The range of MgATP levels was limited due to the high background absorbance. A more complete analysis of the second phase of the reaction was facilitated by quenching the enzymatic reaction by the addition of EDTA in excess of the total Mg^{2+} concentration after a substantial increase in A_{240} had been observed. EDTA was also added to the reference cuvette as there was a significant A_{240} from the EDTA alone. After quenching, various amounts of acid (HCl or DCl) or base (NaOH or NaOD) were added to the reaction cuvette to obtain the desired pH (D). The pH(D) values for which this decrease in A_{240} was monitored in this manner ranged from 3.5 to 11.

Sodium Borohydride Reductions. Reductions were carried out by a modified procedure of Hartman and Wold (1967) at 4 °C in an ice bath with continuous pH monitoring. Stock solutions (0.66 M, 25 mg/mL) were prepared by dissolving $NaBH_4$ in cold H_2O and were used within 30 min of preparation. Prior to reduction, pyruvate kinase was removed from the TSP reaction mixture by ultrafiltration using an Amicon YM 30 membrane. A 10-fold excess of $NaBH_4$ was added dropwise to ~1 mL of reaction mixture, and the pH was maintained between 7.5 and 8 throughout the reduction. After 15 min, the excess hydride was removed as H_2 by lowering the pH to 6.5. The concentration of D-2PGA was determined enzymatically by using assays containing yeast enolase, pyruvate kinase, lactate dehydrogenase, ADP, Mg^{2+}, K^+, NADH, and Hepes, pH 7.5. Triplicate determinations were performed for each reduction.

^{31}P NMR. Spectra were obtained on a Bruker AM-400 wide-bore spectrometer operating at 162 MHz for ^{31}P. Sample temperature within the magnet was maintained with an FTS Systems Air Jet variable-temperature unit. Reaction mixtures of sufficient volume to allow use of 10-mm NMR tubes were prepared with various H_2O/D_2O compositions and equilibrated at the desired temperature. D_2O was required for both the deuterium lock and to slow down the tautomerization reaction. After an initial spectrum was obtained, the sample was warmed to room temperature and lyophilized pyruvate kinase was added to initiate the reaction. After ~2 min, the solution was chilled and an excess of EDTA was added to quench the enzymatic reaction and to ensure narrow line widths. After allowing for temperature equilibration (~2 min) in the magnet, spectra were obtained at various time intervals. Proton decoupling was accomplished, when required, by using composite pulse decoupling in order to avoid sample heating.

Enolase Kinetic Assays. The dehydration of D-2PGA catalyzed by enolase was monitored, unless otherwise state, by using a coupled assay where PEP was converted to pyruvate by pyruvate kinase, and pyruvate in turn was reduced to lactate by lactate dehydrogenase with the concomitant oxidation of NADH [ϵ_{340} = 6220 M^{-1} cm^{-1} (Horecker & Kornberg, 1948)]. All assays were run in 3-mL volumes at pH(D) 7.2 and 25 °C. Cuvettes typically contained 100 mM Hepes, 100 mM potassium acetate, 10 mM magnesium acetate, 133 μM NADH, 1.5 mM ADP, 40 units of pyruvate kinase, and 110 units of lactate dehydrogenase. Depending on the experiment, various amounts of inhibitor, D-2PGA, and enolase were also added. With noted exceptions, reactions were initiated by the addition of D-2PGA. All stock solutions were filtered through a Millex-HA 0.45-μm filter unit (Millipore Corp.) to remove any particulate matter.

Inhibition of Yeast Enolase by the Enol of TSP. The phosphorylated enol of TSP was generated in D_2O as described above. When a sufficient increase in absorbance at 240 nm was obtained, as monitored with a Cary 118 dual-beam spectrophotometer, the phosphorylation reaction was quenched by the addition of EDTA. Aliquots were removed and immediately added to an enolase-coupled assay, also in D_2O. A second spectrophotometer (Beckman DU) was used for these assays. After the residual β-hydroxypyruvate was reduced to glycerate by lactate dehydrogenase, initial velocities for the enolase reaction were obtained by the addition of Mg^{2+} and either D-2PGA (preincubation of enolase and enol) or enolase (*no* preincubation of enolase and enol). The concentration of enol, and thus the extinction coefficient, was estimated by the difference in β-hydroxypyruvate concentration as a result of the pyruvate kinase reaction. When bursts were observed, the velocity was calculated from the final steady-state rate. The apparent K_i of the hydrated aldehyde form of D-TSP was determined in a similar fashion, except that aliquots were not removed until the tautomerization reaction was judged complete on the basis of no further change in the absorbance at 240 nm. Uninhibited initial velocities were also determined, again by the addition of Mg^{2+} and either D-2PGA or enolase to initiate the reaction. The Michaelis constant for D-2PGA under these conditions was determined by varying the concentration of D-2PGA at a fixed free Mg^{2+} level of 8.5 mM.

Cadmium Inhibition of Yeast Enolase. Initial velocities to determine the inhibition by Cd^{2+} of enolase were obtained by directly following the increase in absorbance at 230 nm due to the formation of PEP. Cuvettes contained the following in 3-mL volumes: 100 mM Hepes, pH 7.4, D-2PGA, and yeast enolase. Various concentrations of Mg^{2+} were added along with several fixed levels of Cd^{2+} to initiate the reactions. In order not to see a rate without added Mg^{2+}, all solutions except magnesium and cadmium were treated with Sigma Chelating Resin (sodium form, dry mesh 50–100) and filtered. The pH was maintained at 7.2 throughout this treatment. Initial velocity patterns were obtained at 60 μM and 1 mM D-2PGA.

Data Analysis. Experimental data were fitted to the following equations by using modified Fortran programs of Cleland (1979). Rate constants for both the enzyme-dependent increase and enzyme-independent decrease in A_{240} were simultaneously determined by fitting the absorbance vs time values at each pH(D) to eq 1, which assumes a first-order process for both the appearance and disappearance of A_{240}.

$$Y = [Ak_1/(k_2 - k_1)](e^{-k_1t} - e^{-k_2t}) + C \quad (1)$$

In eq 1, Y is the absorbance at time t, A is the estimated extinction coefficient for the phosphorylated enol of TSP,[2] k_1

[2] The apparent extinction coefficient for the phosphorylated enol at 240 nm was estimated to be between 8 and 9.5 mM^{-1}, both from simultaneous solving of a double-exponential equation similar to eq 1 where A is treated as an unknown and from the difference spectrum of the Mg–enolate of TSP (Spring & Wold, 1971a).

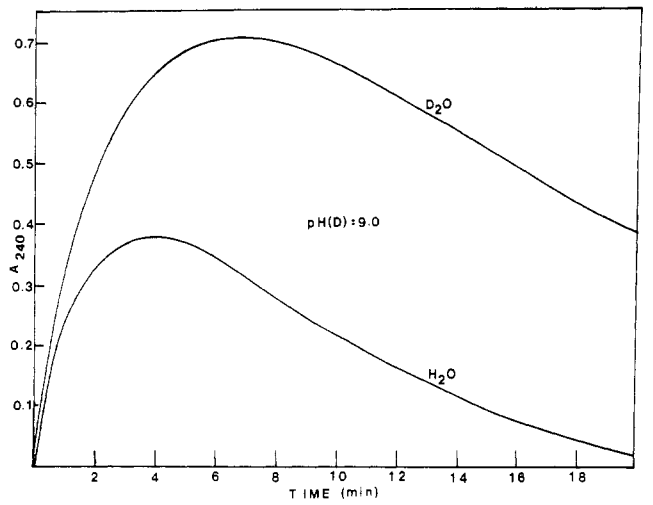


FIGURE 1: Progress curves for the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate in H_2O and D_2O. Rate constants for both phases of each reaction were obtained by fitting the data to eq 1. MgATP was 167 μM in both the reaction and reference cuvettes, and β-hydroxypyruvate was 1 mM.

and k_2 are the desired rate constants, and C is a constant term. When only the enzyme-independent decrease in A_{240} was followed using the EDTA quench–pH jump method, the rate constant at each pH(D) was determined by fitting the absorbance vs time values to eq 2. Reciprocal initial velocites

$$Y = Ae^{-kt} \quad (2)$$

from initial velocity patterns were plotted vs reciprocal substrate concentrations, and the data were fitted to eq 3, which describes a sequential (random or ordered) addition of substrates to enzyme. The pH(D) profile for the initial reaction,

$$\log v = \log [VAB/(K_aB + K_bA + AB + K_{ia}K_b)] \quad (3)$$

in which the log of the parameter decreased both above pK_2 with a slope of −1 and below pK_1 with a slope of 1, was determined by fitting the k_1 values at each pH(D) value to eq 4, where H is the hydrogen ion concentration, K_1 and K_2 are

$$\log k_1 = \log [C/(1 + H/K_1 + K_2/H)] \quad (4)$$

the acid dissociation constants, and C is the plateau value of the parameter. The pH(D) profiles for the tautomerization reaction, where a plateau at both high and low pH(D) was observed, were analyzed by fitting the k_2 values at each pH(D) to eq 5, where Y_L and Y_H are the plateau values at low and

$$\log k_2 = \log [(Y_L + Y_HK/H)/(1 + K/H)] \quad (5)$$

high pH(D), respectively, and K is the acid dissociation constant. Individual saturation curves were fitted to eq 6.

$$v = VA/(K_m + A) \quad (6)$$

Apparent K_i values for both the enol and the hydrated aldehyde forms of TSP were calculated from eq 7, which was derived from eq 6 and 8 (vide infra). In eq 7, I is the inhibitor

$$\text{apparent } K_i = I/[(1 + A/K_m)(v_0/v_i - 1)] \quad (7)$$

concentration, A is the concentration of D-2PGA (fixed at 100 μM), K_m is the Michaelis constant for D-2PGA determined from eq 6 (64 μM), and v_0 and v_i are the uninhibited and inhibited initial velocities, respectively.

RESULTS

Direct Observation of the Pyruvate Kinase Reaction. Figure 1 is a typical example of the change in A_{240} vs time seen at the different pH(D) values examined. By fitting these data to eq 1, the rate constants for both phases of this reaction were obtained. The rate constant for the initial increase in A_{240}, k_1, was found to be directly proportional to the amount of enzyme present, whereas the rate constant for the subsequent decrease in A_{240}, k_2, was found to be independent of the amount of enzyme present.

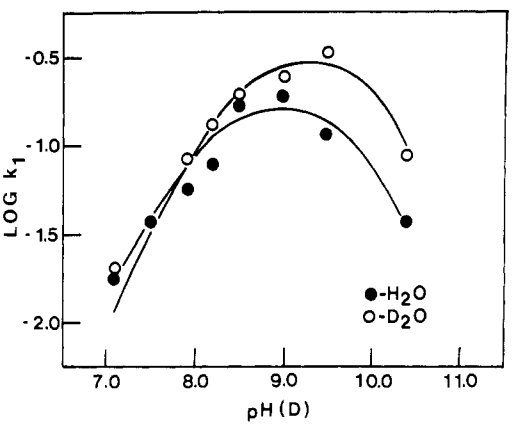


FIGURE 2: Variation of the phosphorylation of β-hydroxypyruvate by pyruvate kinase with pH(D). Closed circles are from reactions in H_2O; open circles are in D_2O. The k_1 values (from eq 1) at each pH(D) were fitted to eq 4, and pK values of 9.7 ± 0.2 and 8.2 ± 0.2 (H_2O) and 9.8 ± 0.4 and 8.7 ± 0.3 (D_2O) were obtained. Units of k_1 are min^{-1}.

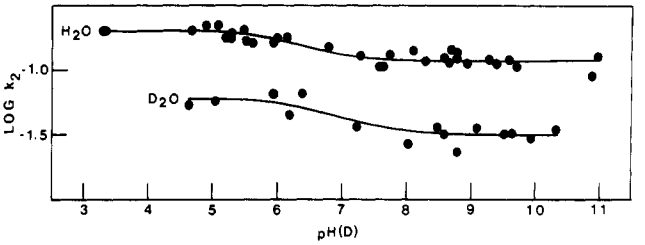


FIGURE 3: Variation of the rate of tautomerization of the enol of TSP to the hydrated aldehyde with pH(D). k_2 values (from eq 2) were determined by the EDTA quench–pH(D) jump method. Top curve is for reactions in H_2O and bottom curve in D_2O. The k_2 values at each pH(D) were fitted to eq 5, and pK values of 6.3 ± 0.3 (H_2O) and 6.7 ± 0.4 (D_2O) were obtained. Units of k_2 are min^{-1}.

Fitting the k_1 values for reactions in H_2O to eq 4 indicated that k_1 decreased above a pK of 9.7 ± 0.2 and below a pK of 8.2 ± 0.2. The k_1 values for reactions in D_2O decreased above a pK of 9.8 ± 0.4 and below a pK of 8.7 ± 0.3 (Figure 2). For reactions in H_2O, C, the plateau value of k_1, is 0.23 ± 0.05 min^{-1}, while for those in D_2O, C has a value of 0.5 ± 0.3 min^{-1}. The solvent deuterium isotope effect on the intial phase of the reaction is the ratio of these C values [$k_1(H_2O)/k_1(D_2O)$], or 0.5 ± 0.3.

Patterns of initial velocities for β-hydroxypyruvate at different levels of MgATP were intersecting, and the data were fitted to eq 3. The only term in the denominator of eq 3 for which a well-defined value was calculated was the cross product $K_{i(MgATP)}K_{\beta\text{-hydroxypyruate}}$ (0.109 ± 0.004). Only approximate values for $K_{i(\beta\text{-hydroxypyruvate})}$ (4 mM) and K_{MgATP} (27 μM) were obtained from the fitted data. When a value of 0.99 mM is used for $K_{i(MgATP)}$ from the phosphorylation of pyruvate (Dougherty & Cleland, 1985), a value of 110 μM is calculated for $K_{\beta\text{-hydroxypyruvate}}$.

The k_2 values determined from either the double exponential fits of the complete A_{240} spectrum or the single exponential fits of only the isolated second phase of the reaction at each pH(D) value were fitted to eq 5. The results for k_2 values from the EDTA quench–pH(D) jump reactions only are shown in

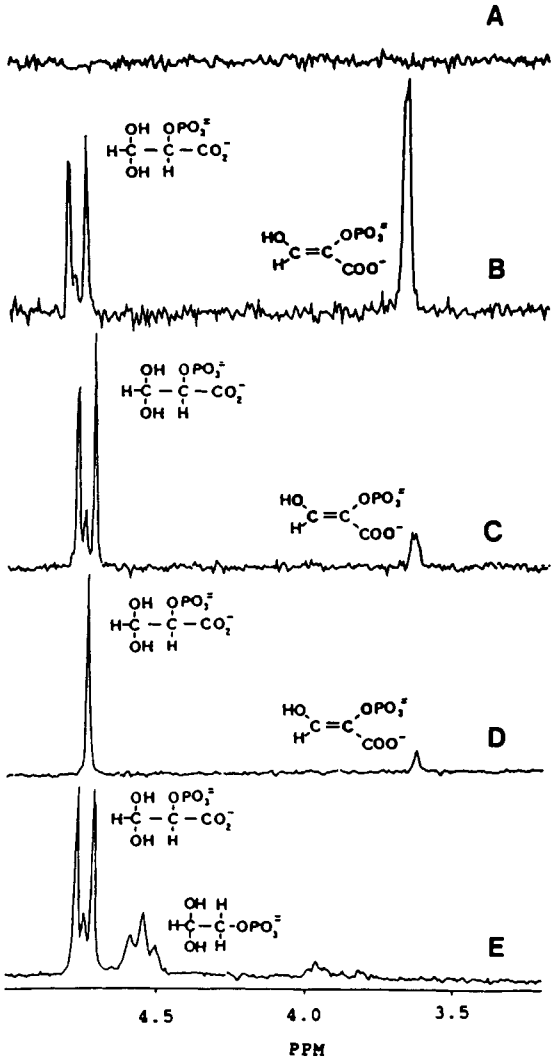


FIGURE 4: ^{31}P NMR spectra for the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate at 5 °C. The reaction mixture contained, in 45% H_2O, 25 mM β-hydroxypyruvate, 25 mM ATP, 37 mM $Mg(OAc)_2$, 50 mM KOAc, and 100 mM Ches, pH 9. Only the downfield region corresponding to phosphomonoesters is shown. The proposed structures of the different species assigned to each resonance are indicated next to the corresponding peak. Spectrum A was obtained prior to the addition of pyruvate kinase and EDTA. Spectrum B was obtained 9 min after the pyruvate kinase reaction was quenched with EDTA. Spectrum C was obtained after an additional 11 min at 25 °C. Spectrum D is the same as spectrum C, except ^1H decoupled. Spectrum E was obtained after the sample was allowed to sit at 25 °C for 3 h.

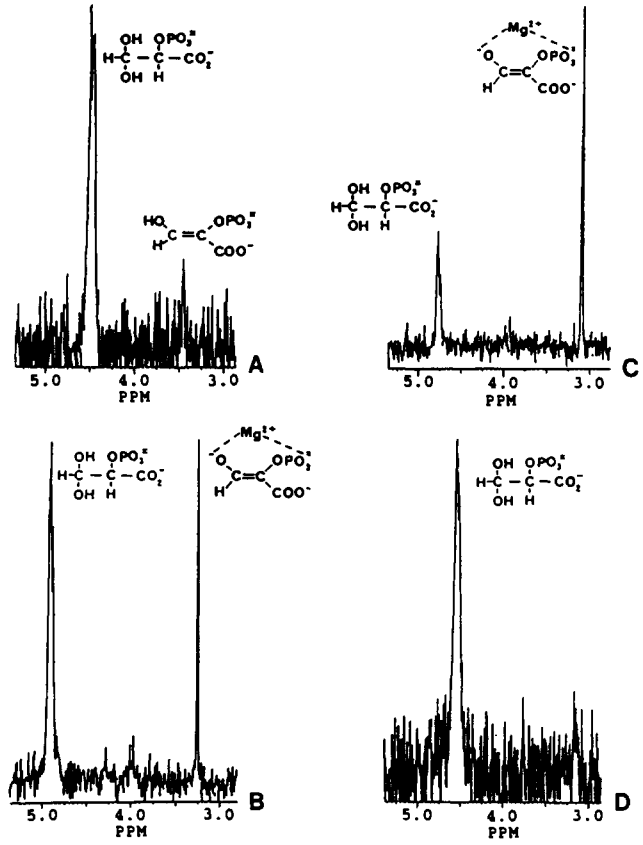


FIGURE 5: ^{31}P NMR spectra for the reversible enolization of D,L-TSP at 25 °C. Proposed structures assigned to each resonance are indicated. The reaction mixture contained, in 45% H_2O, approximately 10 mM TSP, 12 mM free Mg^{2+}, and 100 mM Ches, pH 9. Spectrum A represents the initial conditions. Spectra B, C, and D were obtained after the pH was adjusted to 13.2, 14, and 8, respectively.

Figure 3. The rate constant for reactions in H_2O, $k_2(H_2O)$, is 0.20 ± 0.01 min^{-1} below a pK of 6.3 ± 0.3, decreasing to 0.117 ± 0.003 above this pK. For reactions in D_2O, $k_2(D_2O)$ is 0.061 ± 0.007 min^{-1} below a pK of 6.7 ± 0.4, decreasing to 0.031 ± 0.002 above this pK. The solvent deuterium isotope effect for the tautomerization reaction is the ratio of $k_2(H_2O)/k_2(D_2O)$ and is 3.3 ± 0.4 at low pH(D) and 3.8 ± 0.3 at high pH(D), giving an average value of 3.6 ± 0.5.

Sodium Borohydride Reductions. It was found that β-hydroxypyruvate decomposed with a half-life of ~65 min at 25 °C and pH 9.2 (the pH optimum for the k_1 portion of the reaction) in the presence of Mg^{2+}. Thus, initial levels of β-hydroxypyruvate were calculated by correcting the initial concentration determined by lactate dehydrogenase end-point assays for the predicted amount of decomposition (typically 2–3%). This value was used to determine the percent of the D isomer of D-2PGA, and thus D-TSP, after $NaBH_4$ reduction of the TSP reaction mixture. From reductions of eight separate pyruvate kinase reactions, an average value of 48 ± 3% D-2PGA was obtained by enolase end-point assay.

^{31}P NMR. The series of spectra shown in Figure 4 were obtained at 5 °C. The only region of the spectra shown is that which corresponds to phosphomonoesters, as the upfield region containing the nucleotide (ATP and ADP) resonances has been omitted for simplicity. Integration showed, however, that the sum of the hydrated TSP and the phosphorylated enol peaks equaled those for the ADP, as expected. The reaction was carried out in 45% H_2O to allow detection of the phosphorus–proton coupling. ^{31}P resonances were observed for the phosphorylated enol of TSP (singlet at 3.65 ppm, relative to 85% phosphoric acid), the hydrated aldehyde form of TSP (doublet at 4.75 ppm), and the decomposition product, glycolaldehyde phosphate (Weiss & Cleland, 1983) (triplet at 4.55 ppm). The series of spectra shown in Figure 5 were obtained at 25 °C with a sample of TSP from which the pyruvate kinase had been removed by ultrafiltration. A resonance at 4.55 ppm was observed for the hydrated aldehyde form of TSP. At pH 13.2, a new resonance, corresponding to the magnesium enolate of TSP, appeared at 3.1 ppm and was of approximately equal intensity to that of the remaining hydrated aldehyde resonance. At pH 14, the resonance at 3.1 ppm was ~3 times that of the remaining hydrated aldehyde. Re-formation of the hydrated aldehyde occurred upon addition of HCl to pH 8, with a corresponding reappearance of the resonance at 4.55 ppm and disappearance of the Mg–enolate resonance at 3.1 ppm.

Inhibition of Yeast Enolase by the Enol of TSP. From three experiments where enolase was preincubated with the enol of TSP, and three where enolase was added last to initiate

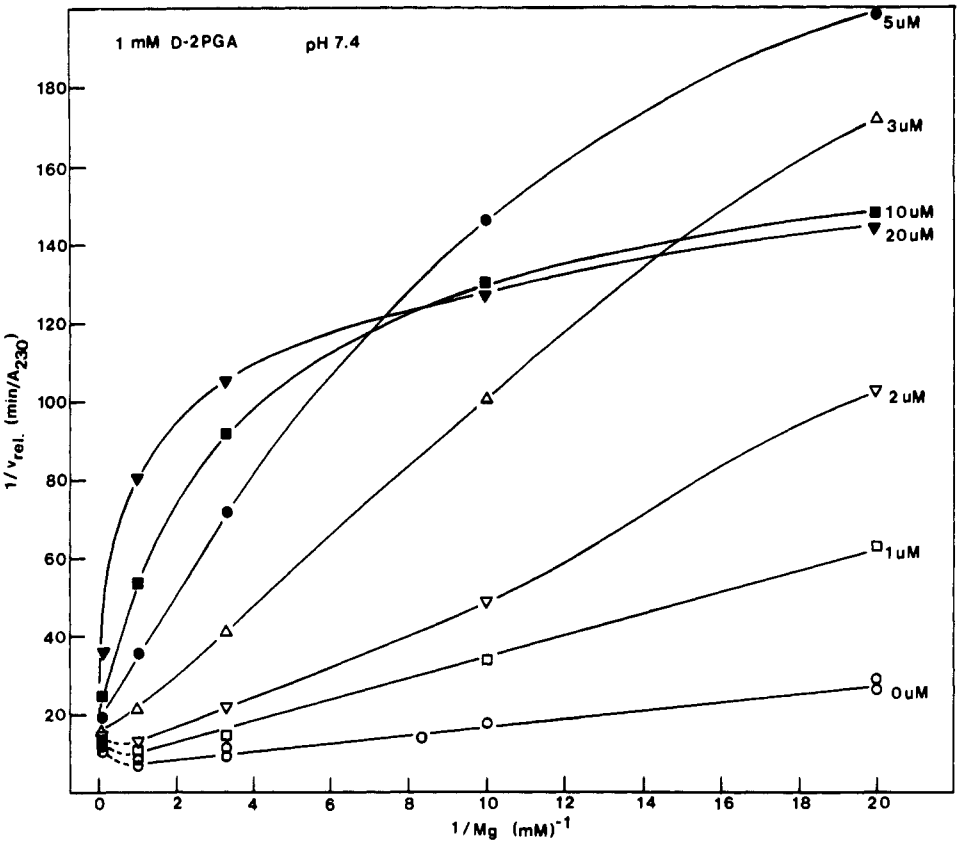


FIGURE 6: Inhibition of yeast enolase by cadmium ion. The concentration of D-2PGA was fixed at 1 mM and is approximately equal to the K_m. The various concentrations of Cd^{2+} are indicated in the right margin. Due to the curvature of the lines, no attempt was made to fit these data. A similar pattern was obtained with 60 μM D-2PGA.

the reaction, an average apparent K_i value of 106 ± 3 nM was obtained. The apparent K_i for the hydrated aldehyde was ~5 μM under the same conditions. When enolase and D-2PGA were added last and there was no preincubation of enzyme and inhibitor, a burst was observed. The half-time for this burst was ~15 s (k = 0.046 s^{-1}), and the initial K_i value was ~200 nM. Conversely, when D-2PGA was added after enzyme and inhibitor had been preincubated for 7 min, a linear steady-state rate was observed. The final steady-state rates for reactions where a burst was observed were the same as the initial steady-state rate for reactions where no burst was observed. Rates of reaction where no inhibitor was present were the same regardless of whether the reaction was initiated by D-2PGA or enolase.

Cadmium Inhibition of Yeast Enolase. A reciprocal plot of the data from the competitive inhibition of yeast enolase by Cd^{2+} vs Mg^{2+} is shown in Figure 6. Due to the curvature of these lines, no attempt was made to fit these data.

DISCUSSION

In order to monitor the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate, we developed a direct spectrophotometric assay. Following the reaction of 240 nm, we expected that a decrease in absorbance would be observed in going from β-hydroxypyruvate (carbonyl conjugated to the carboxyl) to TSP (no conjugation between hydrated aldehyde and carboxyl). The balancing of the background A_{240} due to the adenine-containing species permitted precise quantitation of absorbance changes. However, an initial increase in A_{240} was observed following the addition of pyruvate kinase, due to formation of the conjugated phosphorylated enol of TSP. The pH dependence of the apparent first-order rate constant for this reaction in H_2O is the same as that for the V/K and

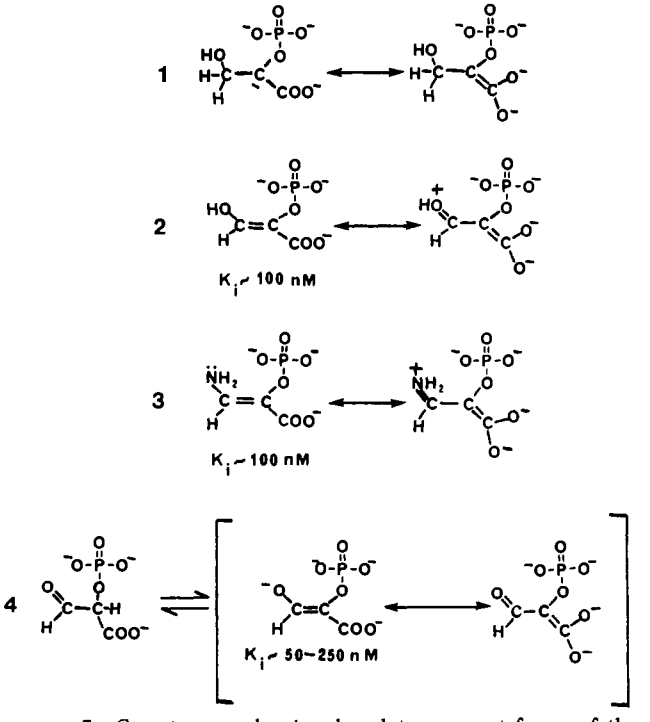


FIGURE 7: Structures and *aci*-carboxylate resonant forms of the intermediate in the enolase reaction, and intermediate analogues. (1) is the carbanion intermediate, (2) is the enol of TSP, (3) is AEP, and (4) is D-TSP. In the case of D-TSP, the aldehyde form is converted to the enolate after being bound to the enzyme.

V values for the pyruvate kinase catalyzed phosphorylation of pyruvate, where pK's of 8.3 and 9.2 were determined (Dougherty & Cleland, 1985).[3] The shift in the low pK on

going from H_2O to D_2O is 0.5 unit, as expected (Schowen, 1977) for a proton on a nitrogen or oxygen. Lysine 269 (E-NH_2) is believed to be the catalytic base responsible for the enolization of pyruvate prior to phosphorylation by MgATP to give PEP (Dougherty & Cleland, 1985), and this is consistent with the observed shift in pK's.[4]

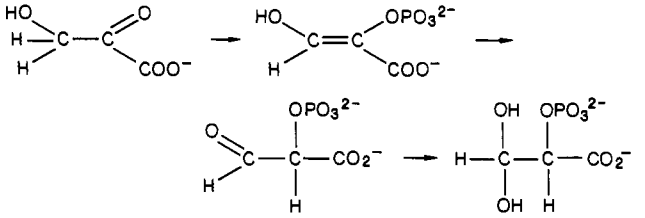
For the steady-state kinetics of the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate to give the enol of TSP, the following values have been assigned for the relevant kinetic constants: $K_{i(MgATP)}$, 0.99 mM (Dougherty & Cleland, 1985); $K_{i(\beta\text{-hydroxypyruvate})}$, 4 mM; K_{MgATP}, 27 μM; $K_{\beta\text{-hydroxypyruvate}}$, 110 μM. The ratio of the true dissociation constant from the binary complex to the Michaelis constant for dissociation from the ternary complex [$K_{i(substrate)}/K_m$) for both substrates is 36. These ratios represent a 36-fold synergism in binding of substrates; that is, the binding of β-hydroxypyruvate lowers K_{MgATP} 36-fold and vice versa. This synergistic effect explains why only the constant term in the denominator could be calculated from eq 3, as the K_m values were too low in comparison to be accurately determined.

The initial increase in absorbance at 240 nm was followed by an exponential decrease. The rate constant for this process, k_2, was independent of enzyme concentration, showing that the reaction was occurring "off" the enzyme; that is, the phosphorylated enol was being released into solution. At first glance, the pH independence of the tautomerization suggests a mechanism in which the low level of enolate in equilibrium with the enol reacts directly with a proton at C-2 in the rate-limiting step. Since the proportion of enolate decreases as the pH is decreased, while the concentration of protons increases, such a mechanism predicts a pH-independent reaction. However, the pK of the enol is unlikely to be less than 14, so that at pH 6, for example, only 10^{-8} of the enol is ionized. While protonation of the oxygen of an enolate will show a bimolecular rate constant of 4×10^{10} M^{-1} s^{-1}, protonation on the carbon is always much slower (Eigen, 1964). The observed rate of tautomerization of 0.2 min^{-1} (3.3×10^{-3} s^{-1}) would require a bimolecular rate constant for protonation of the enolate at C-2 of 3×10^{11} M^{-1} s^{-1}, which is clearly impossible. We conclude that the tautomerization involves concerted proton transfers, with water as the likely donor at C-2 and acceptor for the enol hydroxyl.

The k_2 values varied above and below the pK of the phosphate group, being 2-fold faster at low pH(D). It should be noted that the pK of 6.3 ± 0.3 for the phosphate group in H_2O increased 0.4 pH unit to 6.7 ± 0.4 in D_2O, as expected (Schowen, 1977).

The average value for the solvent deuterium isotope effect on the tautomerization reaction of 3.6 ± 0.5 is of the magnitude one would expect for the proposed chemistry. Values for K_{H_2O}/k_{D_2O} have been reported for the tautomerization of both enolbutyrate to 2-oxobutyrate (4.1) (Gonzalez & Andrea, 1988) and enolpyruvate to pyruvate (~6) (Kuo et al., 1979).

The fact that the tautomerization reaction occurs in solution suggested that the stereochemistry of the resulting tautomer should be racemic, as the probability of collapse of the phosphorylated enol to D- or L-TSP is equal. This hypothesis was confirmed, as 50% D-2PGA, and therefore D-TSP, was obtained from $NaBH_4$ reductions of the reaction mixture.[5] Thus, the eventual product of the pyruvate kinase catalyzed phosphorylation of β-hydroxypyruvate is D,L-TSP, and the overall reaction can be written as



Having proposed that the initial product of this reaction is the enol of TSP, we sought further evidence for its existance from ^{31}P NMR. Two distinct resonances were observed when the reaction was carried out at 5 °C in 45% H_2O, a transient one and a second more stable one. Over time, the former resonance disappeared and was quantitatively converted to the latter resonance ~1 ppm downfield. Only the latter resonance was previously observed by Ash et al. (1984) because their spectrum was obtained after 2 h at room temperature. Because

[3] Although the enzymatic reaction is not strictly first order because neither substrate was well below its K_m value, the reaction is approximately a first-order one because of the rapid approach to equilibrium before a large portion of either substrate is consumed. Note that the equilibrium constant for the phosphorylation of β-hydroxypyruvate, estimated to be 0.1 at pH 9 from the ^{31}P NMR studies, is much higher than that for the phosphorylation of pyruvate, which is 3×10^{-3} at pH 9 (McQuate & Utter, 1959). This is presumably because of the greater ease with which β-hydroxypyruvate is enolized.

[4] The apparent shift in the high pK, however, is only 0.1 unit, although this difference is not well-defined as the standard error on this value is ±0.5. An explanation for this small shift, as well as the inverse solvent deuterium isotope effect on k_1 of 0.5 ± 0.3, is offered below; however, this is rather tenuous, given the precision of the experimental data. Kiick et al. (1987) recently suggested, on the basis of pK_i profiles with different metal ions that support the pyruvate kinase catalyzed decarboxylation of oxaloacetate, that the high pK may not be due to water coordinated to enzyme-bound Mg^{2+} as proposed by Dougherty and Cleland (1985). Although no alternative base was suggested, there are a number of observations consistent with a sulfhydryl as the group with this high pK. The 0.1-unit shift in the pK value is in good agreement with the expected shift of 0.18 calculated for a sulfhydryl group. The inverse solvent isotope effect suggests that a group with a fractionation factor of ~0.5 has its proton transferred during the reaction. The only reasonable group with a fractionation factor so far below unity is a sulfhydryl (Cleland, 1980). Further, Dougherty and Cleland (1985) found that in the presence of 20% propylene glycol the pK at 9.2 shifted to outside the accessible pH range. This was taken as evidence for a neutral acid, which was concluded to be water coordinated to enzyme-bound metal; however, this is also consistent with a sulfhydryl group. From sequence and X-ray crystallographic data for pyruvate kinase from cat and chicken muscle, rat liver, and yeast, cysteine 325 is conserved in all cases and is assigned a position directly adjacent to part of the active site (Muirhead et al., 1986). Also, from a three-dimensional α-carbon drawing of pyruvate kinase with PEP and metal bound, it appears that cysteine 325 is very near the enzyme-bound Mg^{2+} (Muirhead et al., 1986). Finally, it has been known for some time that pyruvate kinase is inactivated by sulfhydryl reagents such as p-mercuribenzoate (Kupiecki & Coon, 1959) and DTNB (Flashner et al., 1972). In the case of DTNB modification, protection against inactivation was achieved under a number of conditions; however, Mg^{2+} was required in all cases, except protection by PEP alone (29% vs 95% for PEP + Mg^{2+}), where significant protection was observed (Flashner et al., 1972). This also suggests that a cysteine, which appears essential for catalytic activity, is near the enzyme-bound metal site. More recently, Rafter and Blair (1987) found that modification of a single cysteine residue with N-ethylmaleimide was accompanied by an irreversible loss of activity. It is possible, then, that a protonated sulfhydryl group with pK ~9.5 is required for pyruvate kinase activity. Above this pK, the extra negative charge in the active site may be responsible for the decrease in both binding and catalysis, as the same pK was seen in both the V and V/K profiles for pyruvate (Dougherty & Cleland, 1985). Whether or not the sulfhydryl group of cysteine 325 (or some other cysteine) must be protonated for activity, it appears that more experiments are necessary to determine the exact nature of this group with the high pK and to understand more fully the chemical mechanism of pyruvate kinase.

[5] The equilibrium for this tautomerization lies well in favor of the aldehyde form, partly because the hydration of the aldehyde to at least 95% (Stubbe & Abeles, 1980) pulls the reaction in that direction.

the solution was 45% H_2O, this final resonance is actually a singlet and a doublet of unequal proportions, the singlet being from the D,L-2-deuterio-TSP (no detectable three-bond P–H coupling) and the doublet being from the D,L-2-protio-TSP (three-bond P–H coupling of 10 Hz). As a result of the large solvent deuterium isotope effect discussed above, there is much more D,L-2-protio-TSP than D,L-2-deuterio-TSP after 9 min. The upfield resonance, which corresponds to the enol of TSP, appears as a very narrow doublet due to the long-range four-bond P–H coupling of ~2 Hz. This, as well as the three-bond coupling of ~10 Hz, is within the expected range for such phosphorus proton couplings (Emsley & Hall, 1976). All resonances became singlets when proton decoupled.[6] TSP is known to decompose rapidly via acid-catalyzed decarboxylation to glycolaldehyde phosphate (Weiss & Cleland, 1983). One would expect that over a long period of time at pH 9 and 25 °C a triplet corresponding to glycolaldehyde phosphate should appear, as the phosphorus is now coupled to two chemically equilvalent protons. After 3 h, such a new resonance did appear, slightly upfield from TSP. Similar resonances from a decomposition product were observed by Ash et al. (1984).

As a prelude to their study of D-TSP as an inhibitor of various enolases, Spring and Wold (1971a,b) demonstrated that the Mg–enolate of TSP could be observed spectrophotometrically at 260 nm. This was accomplished by titrating a 35 μM solution of D-TSP to pH 12 in the presence of 1 mM Mg^{2+}. At pH 12 without Mg^{2+}, and pH 9 with Mg^{2+}, no absorbance at 260 nm was observed. We have reversibly generated the Mg–enolate of TSP and shown it to have a ^{31}P resonance ~1.5 ppm upfield from TSP. It is this Mg–enolate which is believed to be the product of the tautomerization reaction catalyzed by enolase on bound D-TSP.

Enolase catalyzes the reversible dehydration of D-2PGA to PEP via a carbanion intermediate (Dinovo & Boyer, 1971; Stubbe & Abeles, 1981). For the dehydration of D-2PGA, a chemical mechanism can be envisioned whereby an enzymic base, proposed to be a sulfhydryl group (Weiss et al., 1987), abstracts the C-2 proton, leading to the formation of the carbanion. The carbanion is likely stabilized by the subsequent formation of the corresponding *aci*-carboxylate, a resonant form of the carbanion with the negative charge delocalized to the carboxyl oxygens. Indeed, a number of compounds that are analogues of such an *aci*-carboxylate intermediate species have recently been found to be very potent inhibitors of yeast enolase. Thus, in the presence of Mg^{2+} (3-hydroxy-2-nitropropyl)phosphonate and phosphonoacetohydroxamate had K_i values of 6 nM and 15 pM, respectively (Anderson et al., 1984).

The apparent K_i value for the enol of TSP of ~100 nM is the same as that reported for the inhibition of yeast enolase by 3-aminoenolpyruvate 2-phosphate (AEP) (Spring & Wold, 1971b). Both of these inhibitors *appear* to bind ~50-fold tighter than the hydrated aldehyde form of TSP, which has an apparent K_i value of ~5 μM. However, it will be the free aldehyde form that is initially bound by enolase, and since the aldehyde is at least 95% hydrated (Stubbe & Abeles, 1980), the real K_i for D-TSP is 250 nM (~50 nM if 99% hydrated). D-TSP is a pseudosubstrate of enolase, since the C-2 proton is abstracted by the thiolate residue, leading to formation of the enzyme-bound Mg–enolate, and presumably this is the actual inhibitory form of D-TSP, as discussed below. From the similarity in structure of these three inhibitors, the tight binding nature of the inhibition is not surprising, as all three resemble the *aci*-carboxylate form of the carbanion intermediate for the enolase reaction in having a trigonal C-2 and a potential metal ligand site attached to C-3 (see Figure 7). Each of the three forms of the inhibitors shown will have a *aci*-carboxylate resonant structure existing to some degree as a result of the C-3 substituent donating electron density to C-3, thereby permitting further delocalization of the C=C electron density to the carboxyl oxygens. The slow binding phases of these inhibitors show different mechanisms. Lane and Hurst (1974) concluded that the AEP bound in a second-order fashion ($E + I \rightleftharpoons EI$). The rate constants for this process were 2.8×10^5 M^{-1} s^{-1} (association) and $<10^{-2}$ s^{-1} (dissociation) at 4 °C. Conversly, the rate of binding of the aldehyde form of D-TSP was limited by a unimolecular step and can be represented by a two-step mechanism:

$$E + TSP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}TSP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}TSP^* \qquad (8)$$

Here E is Mg–enolase and E–TSP* is the enzyme-bound Mg enolate of TSP. At 4 °C, the rate constants k_3 and k_4 were 0.19 s^{-1} and 0.01 s^{-1}, respectively, and the overall binding constant was calculated to be 4 μM at 4 °C (Lane & Hurst, 1974). This latter value is in good agreement with both the apparent K_i of ~5 μM determined here and the value of 15 μM reported by Spring and Wold (1971b).

The enol of TSP shows slow binding behavior since the enzyme must overcome a sizable kinetic barrier in order to bind a molecule resembling what it would normally only be poised to have bound as an intermediate, *after* forming a tight initial complex with the inhibitor. The apparent K_i value for this initial complex is 200 nM. However, enolase presumably binds AEP to form a weak initial complex which was undetected by stopped-flow and temperature-jump kinetic methods (Lane & Hurst, 1974). The slow binding behavior of AEP is the subsequent tightening of the binding and appears as a first-order process.

Since an intermediate that is not released from the enzyme has a low k_{off} rate constant, it must have a corresponding slow k_{on} rate constant, or it will be so stable that it cannot decompose to either substrate or product on the enzyme; that is, it will exist in a deep hole in the free energy profile. Thus analogues of such intermediates should also show low k_{on} rate constants, as we observed for the enol of TSP.

The relatively large value of k_3 reported by Lane and Hurst (1974) for the binding of D-TSP further suggests that enolase catalyzes the enolization of D-TSP by proton removal from C-2. By catalyzing this process, the sulfhydryl group is in the correct protonation state for binding of an intermediate analogue, the enolate of TSP. Given the enol of TSP directly, however, the enzyme may be in the incorrect protonation state to permit rapid binding. These differences can be better envisioned in terms of the free energy profile for enolase. D-TSP binds, as does D-2PGA, at a presumably low energy level and is able to proceed to the intermediate much easier than the enol of TSP, which must bind at a higher energy level. This apparently slows the rate of binding >4-fold (the enol of TSP had $k = 0.046$ s^{-1} at 25 °C vs D-TSP which had $k_3 = 0.19$ s^{-1} at 4 °C).

Having previously concluded that a sulfhydryl group was at the active site of yeast and muscle enolase (Weiss et al., 1987), we sought to investigate the possible interaction of Cd^{2+} with this residue in yeast enolase. At both low (60 μM, equal

[6] The phosphorylated enol of TSP resonated at 3.65 ppm in these studies. This is considerably downfield from the peaks of PEP (−1 ppm), fluoromethyl-PEP [−3.56 ppm (Wirsching & O'Leary, 1988)], and fluoro-PEP (−1.9 and −4.1 ppm for the *Z* and *E* isomers). The reason for the variation in chemical shifts among these compounds is not known.

to K_m) and high (1 mM) D-2PGA levels, reciprocal plots of $1/v$ vs $1/[Mg^{2+}]$ were hyperbolic at high Cd^{2+} concentrations. This is expected since Cd^{2+} will support the enolase reaction in place of Mg^{2+} (Spencer & Brewer, 1984). Cd^{2+} also appears to be a potent inhibitor vs Mg^{2+}. The sigmoidal pattern of the lines in Figure 6 suggests a high level of cooperativity and that, at 60 μM D-2PGA, Cd^{2+} has replaced Mg^{2+} at a level some 10-fold lower than that required at 1 mM D-2PGA. Spencer and Brewer (1984) suggest that the "structural" Cd^{2+} is bound more tightly than the "catalytic" Cd^{2+}, as they observed similar sigmoidal patterns for the titration of D-TSP–enolase with Cd^{2+}. Despite these results, evidence neither in favor of nor against any interaction between Cd^{2+} and the proposed sulfhydryl group is apparent.

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Registry No. DL-TSP, 118455-76-8; D-2PGA, 3443-57-0; TSP enol, 118377-06-3; Cd, 7440-43-9; pyruvate kinase, 9001-59-6; β-hydroxypyruvate, 1113-60-6; enolase, 9014-08-8.

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