Enolpyruvate: Chemical Determination as a Pyruvate Kinase Intermediate†

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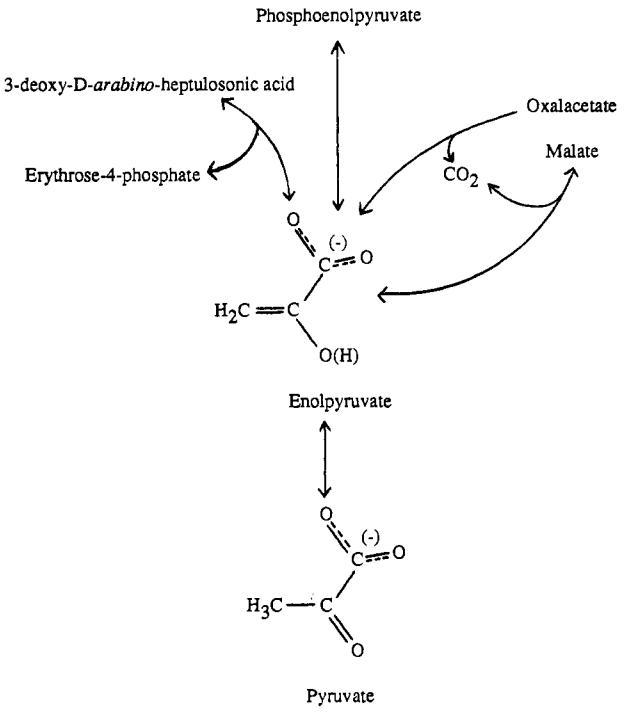
ABSTRACT: Despite many studies suggesting the role of enolpyruvate as a bound intermediate in the pyruvate kinase reaction, direct evidence for it has been lacking. By use of a combination of chemical trapping and isolation of a derivative, significant amounts of enzyme-bound enolpyruvate have now been demonstrated. The method distinguishes enolpyruvate from pyruvate. It is based on reaction of bromine with enolpyruvate in acid, derivatization of formed bromopyruvate with thionitrobenzoate, and resolution by reversed-phase HPLC of the thioether derivative. As little as 10 pmol of the thioether derivative could be quantitated reliably. With this method, the internal equilibria, including the E·ATP·enolpyruvate intermediate, have been determined. Enzyme–enolpyruvate concentration was shown to be pH-dependent. Phosphoenolpyruvate also reacts with bromine to form bromopyruvate. To quantitate enolpyruvate specifically in a background of phosphoenolpyruvate, advantage was taken of phosphoenolpyruvate's much greater stability in acid. When bromine was added 10 min after the acid quench, ketonization of enolpyruvate was complete, and only phosphoenolpyruvate was measured. Enolpyruvate is thus determined by difference between the bromopyruvate measured with and without delayed bromine addition.

Enolpyruvate is chemically implied as the intermediate in substitution reactions catalyzed by enzymes that use phosphoenolpyruvate in the formation of oxalacetate, malate, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate, etc. Detection of enolpyruvate bound to any of these enzymes has not yet been reported. In the case of pyruvate kinase

$$ADP^{3-} + PEP^{2-} + H^+ \rightleftharpoons \text{pyruvate}^- + ATP^{4-} \quad (1)$$

phospho transfer proceeds through a single transition state (Hasset et al., 1985) while a concerted H-/-PO_3^{2-} allylic substitution mechanism is unlikely as evidence accumulates in favor of a two-step mechanism. The two-step mechanism is supported by observations such as ATP-independent enolization of pyruvate and alternative kinase substrates [reviewed by Kayne (1974)]. Furthermore, enolpyruvate generated by action of a phosphatase on PEP is ketonized by pyruvate kinase in the presence of K^+ and Mg^{2+}, the normal cofactors consistent with the catalysis of proton exchange in pyruvate, although the rate is somewhat less than that required for pyruvate formation from ADP + PEP (Kuo & Rose, 1978; Kuo et al., 1979). The detection of enolpyruvate as an enzyme-bound intermediate in the pyruvate kinase reaction should resolve remaining ambiguities as to the chemical mechanism. It would relate the "kinase" to the family of enzymes in which PEP, not pyruvate, is presumed to be the source of enolpyruvate for elongation by addition of CO_2 or aldehydes (Scheme I).

Scheme I



Considering the importance suggested for enolpyruvate in CO_2 fixation, in biosynthesis, and in degradation and the use of ketonization to drive the conversion of ADP to ATP in glycolysis, further attention to the detection, quantitation, and characteristics of enzyme-bound enolpyruvate seems long overdue. To this end a generally applicable method is presented and applied to rabbit muscle pyruvate kinase (EC 2.7.1.40). Bromine in acid is used to trap enolpyruvate that is liberated upon denaturation of the enzyme. This technique makes use of the high reactivity of enolpyruvate with bromine compared with a slow rate of ketonization to pyruvate or spontaneous generation of enolpyruvate from pyruvate. Bromopyruvate is stable in acid and only very slowly hydrolyzed at neutral pH in which it readily reacts with thiols. The thiol ether derivative of the chromophore 5-thio-2-nitrobenzoate provides a product that can be analyzed (ϵ_{342nm} = 9559 cm^2 mol^{-1}, in 0.1 N HCl) upon isolation by reversed-phase HPLC. By this method we have determined the separate

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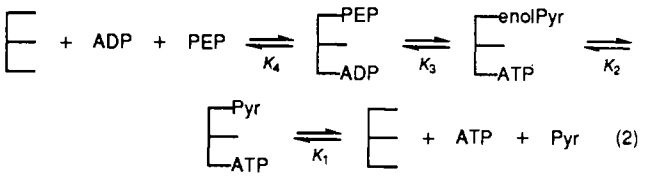
kinase (K_3) and keto–enol (K_2) internal equilibria of the ternary substrate complex of pyruvate kinase:



Materials and Methods

Enolpyruvate Measurements by Formation and Analysis of 3-Bromopyruvate. A general summary of the method is presented here followed by a more detailed account of its application to enolpyruvate measurements in pyruvate kinase:

(I) Enzyme is added to a solution containing substrates, cofactors, buffer components, etc. to form enzyme–enolpyruvate.

(II) Enzyme–enolpyruvate is then added to a rapidly mixing acid-quench solution saturated with liquid bromine.[1]

(III) Bromine and liberated enolpyruvate react to form bromopyruvic acid, and excess bromine is removed under a stream of nitrogen gas.

(IV) Thionitrobenzoate (TNB) is added to the neutralized bromopyruvate solution to quantitatively convert bromopyruvate to TNB-pyruvate.

(V) TNB-pyruvate is measured by absorbance at 342 nm following separation by reversed-phase HPLC.[2]

An aliquot (20–100 μL) of reaction mixture, 0.5–1 mM in pyruvate kinase, is added with rapid vortex mixing to an equal volume of 2 M $HClO_4$ saturated with liquid bromine. After 30 s, during which time the brown bromine color was seen to persist, the excess bromine was rapidly and efficiently removed in a stream of nitrogen gas in the fume hood. 3-Bromopyruvic acid is formed very rapidly from enolpyruvate and from PEP but only slowly from pyruvate under the quench conditions, $k_{enolization} \approx 4 \times 10^{-7}$ s^{-1}, pH ~0.5 at room temperature. Catalytic enzyme concentrations were used in parallel experiments to assess the background contributions to measured bromopyruvate from free substrates and buffer components. Dilution factors and recoveries were determined by use of trace quantities of [2-^{3}H]acetate and radioactive counting of aliquots from the samples before and after acid quench and neutralization. A correction for PEP is made with a second aliquot quenched with $HClO_4$ without bromine. After 10 min (ca. six half-times for the ketonization of enolpyruvate under these conditions), 1 μL of liquid bromine is added for reaction with the PEP, which was otherwise stable under these conditions. In addition, PEP is assayed independently as detailed below.

Quenched solutions are centrifuged to remove precipitated protein, and the supernatant is neutralized (pH ~7) with 2.5 M K_2CO_3 and stored on ice. 5-Thio-2-nitrobenzoic acid (TNB) is added (the greater of 0.25 mM or a 3-fold excess of expected enolpyruvate plus phosphoenolpyruvate) to form the pyruvyl-TNB adduct (Yun & Suelter, 1978), at least 20 min at room temperature being allowed for reaction. The UV spectra of various adducts and DTNB itself being very similar, the analysis required separation of the reaction components. This was achieved by reversed-phase HPLC. Integrated peak intensities from experimental samples were compared to those of a standard curve generated with authentic BrPyr solutions of known concentration (assayed with lactic dehydrogenase/NADH) and chromatographed under identical conditions.

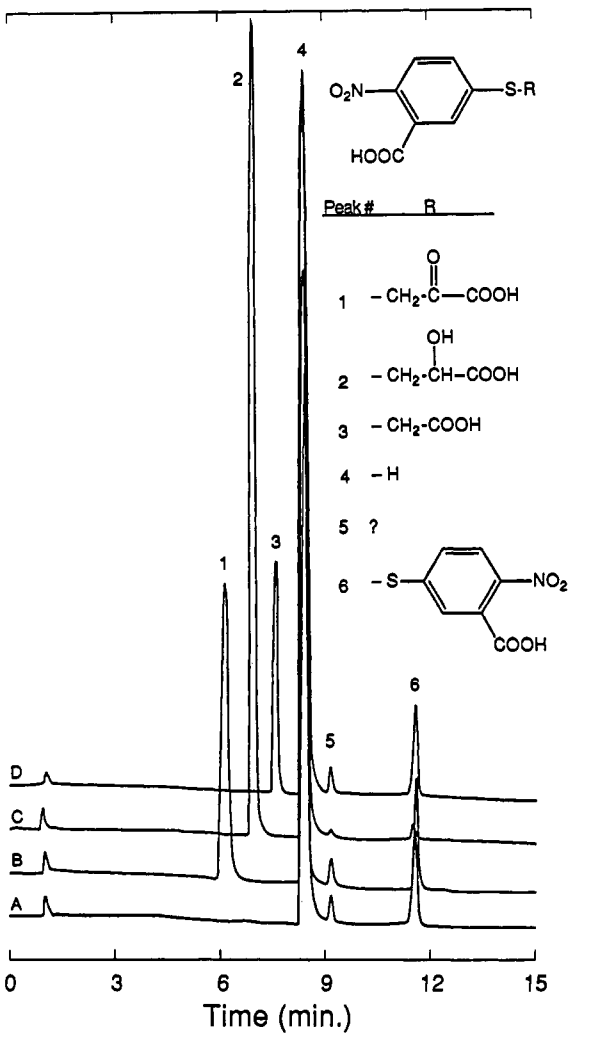


FIGURE 1: HPLC traces of (A) TNB, (B) TNB-pyruvate, (C) TNB-lactate, and (D) TNB-acetate. TNB and DTNB, eluting at 8.5 and 11.6 min, respectively, appear in all the chromatograms with a minor contaminant eluting at 9.2 min. Elution times for TNB-pyruvate, TNB-lactate, and TNB-acetate are 6.2, 7.0, and 7.7 min, respectively. Borohydride treatment of a TNB-pyruvate sample identical with that chromatographed in (B) results in a quantitative reduction of TNB-pyruvate to TNB-lactate as well as a reduction of the DTNB peak to TNB.

As shown in Figure 1, baseline resolution was obtained for the following TNB compounds listed in order of elution: TNB-pyruvate, TNB-lactate, TNB-acetate, TNB, and DTNB. A Waters PICO-TAG column was used at a flow rate of 1 mL/min with a 13-min linear gradient from 5% acetonitrile–95% buffer A (10 mM H_3PO_4, 0.1 M NaCl, pH 2.2) to 70% acetonitrile–30% buffer A. Peaks were detected at a wavelength of 342 nm on a Varian Vari-Chrom detector and integrated with a Hewlet-Packard 3390A integrator.

Assays of Pyruvate, PEP, ATP, and ADP. LDH and NADH were used to measure pyruvate. ATP was measured in the presence of glucose, NADP, hexokinase, and glucose-6-phosphate dehydrogenase. ADP and PEP were measured after high concentrations of pyruvate were destroyed with hydrogen peroxide (100 mM H_2O_2 for 0.5 h at neutral pH) and then by use of LDH, PK, and NADH in a coupled assay in the presence of excess PEP or ADP, respectively. ATP and ADP were also assayed by ion-paired reversed-phase HPLC with a 7-min gradient from 95% buffer B–5% buffer C to 40%

[1] Phosphoenolpyruvate (PEP) also reacts with bromine to form bromopyruvate. PEP, if present, can be determined in a parallel experiment by allowing the ketonization of enolpyruvate for a sufficient time before addition of bromine to the acid-quenched solution.

[2] Appropriate control experiments should be performed.

buffer B–60% buffer C run through a Waters PICO-TAG column at a flow rate of 1 mL/min. Buffer B contained 0.1 M KH_2PO_4, 2 mM KOH, and 5 mM tetrabutylammonium phosphate (Kodak). Buffer C contained a 7:3 methanol–water (v/v) mixture. The chromatogram peaks were quantitated by following the optical density at 259 nm (ϵ_{259} = 15 400 cm^2 mol^{-1}) on a Varian Vari-Chrom detector and integrating the signals with a Hewlet-Packard 3390A integrator. Inorganic phosphate and pyruvate were eluted in the void volume followed by PEP, AMP, ADP, and ATP. Baseline resolution of A_{259nm} peaks was achieved throughout the chromatogram. The same system was used to verify the purity of AMPPCP, which nearly coelutes with ATP.

Materials. Lithium pyruvate monohydrate was prepared by neutralizing pyruvic acid (Aldrich) with lithium hydroxide and recrystallizing three times from hot water (Dickens & Williams, 1958). The salt was verified by IR spectroscopy (Bellamy & Williams, 1958).

ATP and ADP were purchased from Sigma Chemical Co. AMPPCP·Li_4 was obtained from Boehringer Mannheim.

5-Thio-2-nitrobenzoic acid (TNB) was prepared by reduction of 5,5′-dithiobis(nitrobenzoic acid) (DTNB) (Boehringer) with 2-mercaptoethanol, as reported by Degani and Patchornik (1971) and Riddles et al. (1979). The twice recrystallized product (mp 116–117 °C, lit. mp 117–118 °C) was stored desiccated at −20 °C. Stock solutions of TNB were stable for several weeks under N_2 in 10 mM triethanolamine and 0.5 mM EDTA, pH 7.5, at 0 °C. Measured extinction coefficients and pK_a values agreed well with reported values (Riddles et al., 1979).

Rabbit muscle pyruvate kinase was obtained from Boehringer Mannheim Biochemicals as a suspension in 3.2 M $(NH_4)_2SO_4$. It was desalted on Sephadex G-25 equilibrated with 100 mM KCl and 10 mM glycine, pH 8.8, and concentrated with pH adjustment in an Amicon Centricon 10 microconcentrator centrifuge tube. Enzyme protein was measured at 279 nm with an extinction coefficient of 0.54 cm^2 mg^{-1} (Bücher & Pfleiderer, 1955) and a subunit molecular weight of 57 000.

Data Analysis: Determination of the Keto–Enol Equilibrium in the Presence of AMPPCP. A simple equilibrium model (eq 3) was used, where [E] represents the subunit

$$E + Pyr \underset{K_1}{\rightleftharpoons} E\cdot Pyr \underset{K_2}{\rightleftharpoons} E\cdot enolPyr \qquad (3)$$

$$K_1 = [E\cdot Pyr]/[E][Pyr];\ K_2 = [E\cdot enolPyr]/[E\cdot Pyr]$$

equivalent concentration of the complex containing the components K^+, Mg^{2+}, and AMPPCP, present in excess of their binding constants (Mildvan & Cohn, 1966). The assumption is made that AMPPCP binds to PK with an affinity similar to that of ATP [reviewed by Yount (1975)]. Experiments were done to measure [E·enolPyr] as a function of total pyruvate concentration (0–20 mM) while the total concentrations of pyruvate kinase (0.5 mM), $MgCl_2$ (10 mM), and AMPPCP (5 mM) were held constant. The quadratic eq 4 was found that expresses [E·enolPyr] as a function of $[E]_t$, $[Pyr]_t$, K_1, and K_2:

$$[E\cdot enolPyr] = \frac{F - \sqrt{F^2 - 4CD}}{2AB^2} \qquad (4)$$

where $A = K_1^{-1}K_2$, $B = 1 + K_2^{-1}$, $C = AB[Pyr]_t$, $D = AB[E]_t$, and $F = 1 + C + D$. The unknown parameters K_1 and K_2 were determined by nonlinear least-squares regression analysis of the data with the BMDP statistical package run on a Vax 8800 computer.

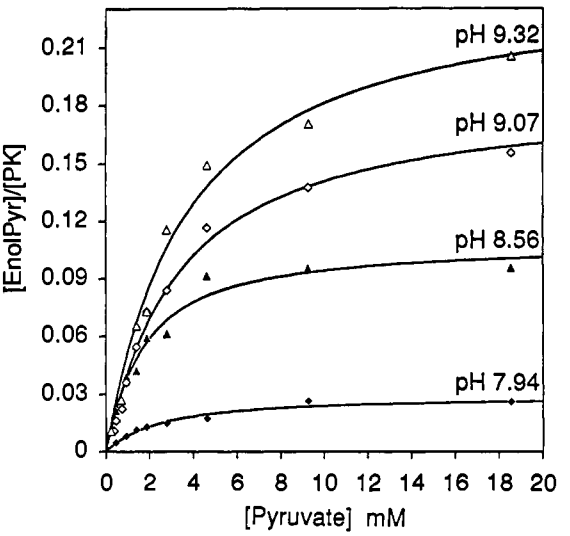


FIGURE 2: [Enolpyruvate] per [enzyme active site] as a function of pH and total [pyruvate]. Experiments were performed at 25 °C with 0.5 mM rabbit muscle pyruvate kinase, 100 mM KCl, 10 mM $MgCl_2$, and 5 mM AMPPCP. The theoretical *lines* are derived from the parameters $1/K_1$ and K_2 determined by nonlinear least-squares analysis of the data (see Materials and Methods and Table I).

Table I: Equilibrium Constants for Pyruvate Binding and Enolization Where E = E·K·Mg·AMPPCP[a]

pH	$1/K_1$ ± SD (mM)	K_2 ± SD
7.94	2.39 ± 0.45	0.030 ± 0.002
8.56	1.62 ± 0.34	0.122 ± 0.008
9.07	3.76 ± 0.51	0.228 ± 0.013
9.32	7.34 ± 0.73	0.322 ± 0.023

[a] Data from Figure 2. $1/K_1$ and K_2 were determined by nonlinear least-squares regression analysis of E·enolPyr measurements as a function of total pyruvate concentrations in the context of the following model:

$$E + Pyr \underset{K_1}{\rightleftharpoons} E\cdot Pyr \underset{K_2}{\rightleftharpoons} E\cdot enolPyr$$

E·enolPyr and pH were measured.

Analysis of data in which pH was varied followed (Cleland, 1979) with

$$\log Y = \log [C/(1 + H/K_a)] \qquad (5)$$

where H is the hydrogen ion concentration, K_a is an acid dissociation constant of a single titratable group, and C is the asymptote of Y at high pH. A three-parameter exponential decay curve of enolpyruvate ketonization in a background of PEP was fit with

$$[BrPyr] = [PEP] + [enolPyr]_{t=0} \exp(-kt) \qquad (6)$$

Results

Observation of Enolpyruvate with Pyruvate Kinase + AMPPCP. In the first attempts to demonstrate enolpyruvate, AMPPCP was used to avoid complications introduced by the formation of PEP, free and bound. With enzyme at 0.5 mM in the presence of KCl (100 mM), $MgCl_2$ (10 mM), and AMPPCP (5 mM), pyruvate was varied from 0 to 20 mM and pH from 9.3 to 7.9. Figure 2 shows the results of these experiments where the enolpyruvate concentration per enzyme active site is plotted as a function of total pyruvate at the indicated pH values. The *lines* are calculated from the fitted parameters (Table I), K_1 and K_2 of eq 3 using eq 4. The keto–enol equilibrium constant, K_2, is also shown in the log–log plot (Figure 3) as a function of pH. Equation 5 was fit to these data with nonlinear least-squares regression analysis where

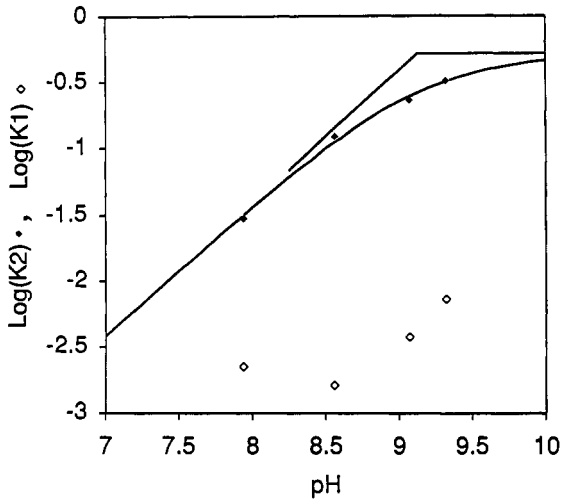


FIGURE 3: log K_2 (closed circles) and log K_1 (open circles) vs pH. The theoretical *curved line* is derived from parameters C (0.518) and K_a (pK_a = 9.13), which were determined by nonlinear least-squares analysis of the K_2 vs $[H^+]$ data with the equation $\log K_2 = \log [C/(1 + [H^+]/K_a)]$. The asymptote and unity slope lines are drawn to aid visual interpretation.

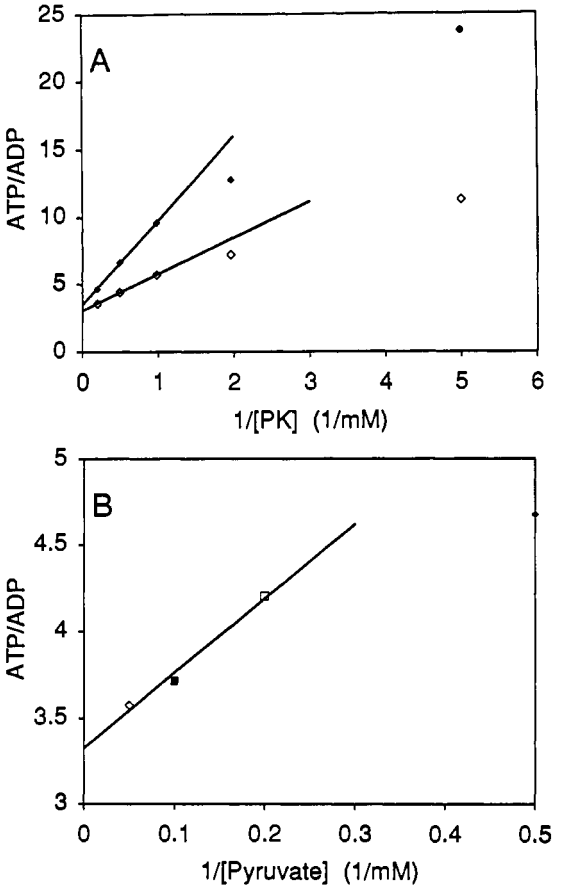


FIGURE 4: ATP:ADP ratios as a function of total pyruvate kinase (A) and pyruvate concentrations (B). Conditions for the experiments were 0.1 M KCl, 15 mM $MgCl_2$, 2 mM ATP (initial), 50 mM glycine, 50 mM glycylglycine, and pH 9.0. In (A), pyruvate kinase was varied in the presence of 20 mM (open symbols) or 2 mM (closed symbols) pyruvate. In (B), pyruvate kinase was fixed at 2.4 mM, and the ATP/ADP ratio was examined as a function of pyruvate concentration. Extrapolations to infinite pyruvate kinase and pyruvate concentrations (shown by the lines) were made by linear regression analysis with the three highest concentration points in each graph.

K_2 was substituted for Y in the equation. K_2 is found to decrease from a value of 0.52 ± 0.08 below a pK_a of 9.13 ± 0.09. Values given are plus or minus asymptotic standard deviations.

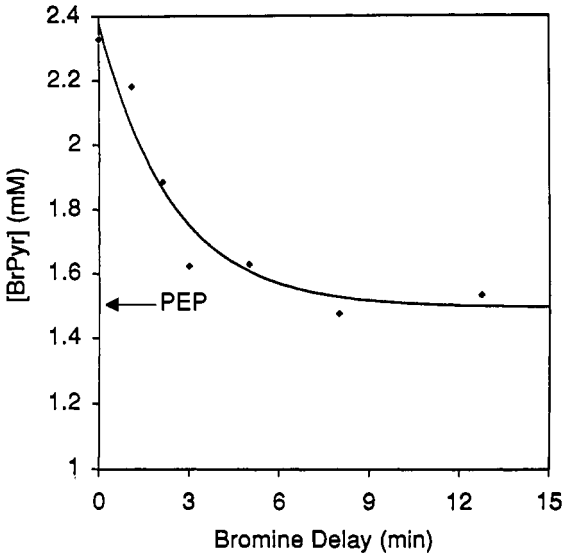
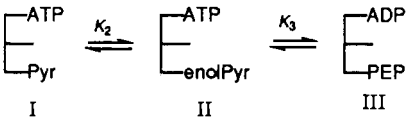


FIGURE 5: Decay kinetics of enolpyruvate under acid-quench conditions. A solution containing ATP, pyruvate, pyruvate kinase, ADP, and PEP at equilibrium (pH 9) was quenched at time zero with perchloric acid (see text for concentrations). Enolpyruvate plus phosphoenolpyruvate concentration was determined by adding bromine at successive times and assaying for bromopyruvate concentration (see Materials and Methods). An exponential decay curve was fit to these data finding a ketonization half-time of 1.66 min. The curve asymptotes to the PEP concentration which remained constant for the duration of the experiment.

Internal Equilibria of Pyruvate Kinase Using ATP:



To determine the two internal equilibria K_2 and K_3, it was necessary to extrapolate the values of total ATP, ADP, and enolpyruvate to infinite concentrations of enzyme and pyruvate so that the ATP and ADP measured would be completely bound. For this purpose it was most convenient to observe the decrease in the ratio of ATP/ADP from the solution value of >10 at low enzyme to the limits shown in Figure 4A as enzyme was increased with either 2 or 20 mM pyruvate, as indicated. This was refined further in Figure 4B in which, at the highest level of enzyme, the pyruvate concentration was additionally varied. The final ratio of ATP/ADP, based on the three extrapolations shown in Figure 4, was 3.3 ± 0.2 at 25 °C with $MgCl_2$ (15 mM), KCl (100 mM), buffer (50 mM glycine, 50 mM glycylglycine, pH 9.0), and total nucleotide (2 mM).

To measure both PEP and enolpyruvate in an incubation, duplicate samples were put into acid. In one sample with Br_2 present in the acid, BrPyr was formed from both PEP and enolpyruvate. In the second sample Br_2 was added after a delay sufficient for the total loss of enolpyruvate to occur. In the latter experiment BrPyr is formed only from phosphoenolpyruvate so the enolpyruvate concentration can be obtained by difference. Figure 5 shows the results of such an experiment where the delay between acid quench and bromine addition was varied up to 13 min. An exponential decay curve (eq 6), fit to these data, indicates the half-time for enolpyruvate ketonization to be 1.66 min under the acid-quench conditions. The time-independent limit gives the PEP concentration as indicated whereas the difference between this value and the measurement at zero time represents the enolpyruvate concentration at time zero. PEP remained constant throughout

Table II: Enolpyruvate Measurements in the Presence of Pyruvate Kinase[a]

	enzymatic assay [PEP] (mM)	HPLC assay [BrPyr] (mM)
(A) acid quench, no bromine	0.24	0
(B) acid quench with bromine	0	0.39
(C) acid quench with addition of bromine after 10-min delay	0	0.24
(d) enolpyruvate concentration [(B) − (C)]		0.15

[a] Additions: 50 mM Gly, 50 mM GlyGly, 100 mM K^+, 15 mM $MgCl_2$, 15 mM Pyr, 2 mM ATP, and 1 mM pyruvate kinase, pH 9.0.

a 0.5-h period in a similar acid-quenched solution of PEP, K^+, Mg^{2+}, and buffer.

Enolpyruvate was measured in the presence of 1 mM pyruvate kinase, 15 mM pyruvate, and 2 mM ATP with this delayed bromine approach. Table II shows the results where 0.15 mM enolpyruvate and 0.24 mM phosphoenolpyruvate were found under these conditions. This enolpyruvate concentration is seen to be in excellent agreement with the enolpyruvate measured in the presence of AMPPCP (Figure 2, pH 9.07) indicating that, for pyruvate kinase, ATP and its diphosphonate analogue promote the enolization of pyruvate to the same extent in the active site of pyruvate kinase.

Discussion

The Method. The method was devised to allow the assay of enolpyruvate in the presence of pyruvate and PEP. The enolpyruvate/pyruvate equilibrium in solution, $\sim 10^{-5}$ at pH 5 to pH 10, and the enolization of free pyruvate under acid-quench conditions account for the small blank of BrPyr. Rapid liberation of bound enolpyruvate is required to avoid losses due to enzymatic action during the course of the acid quench. The small scatter and good consistency of the data shown in Figures 2–5 suggest this condition was satisfied. Reaction of BrPyr with TNB was quantitative under our reaction conditions, resulting in a high A_{340} derivative, readily identified and quantitated by HPLC.

It was important to avoid local basicity when the perchloric acid-quenched samples are being neutralized. Extensive hydrolysis of the BrPyr during neutralization with KOH resulted in low, nonreproducible values. This was avoided with K_2CO_3, which evolves the buffering H_2CO_3 which slowly evolves CO_2 at neutral pH.

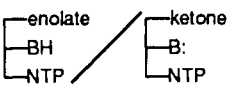
Although the method does not directly distinguish between enolpyruvate and PEP, this was no problem when AMPPCP was used and would be *directly* applicable to investigate the presence and amount of enolpyruvate in enzymatic reactions such as transcarboxylase, pyruvate carboxylase, OAA decarboxylase, malate decarboxylase, and others. Indirect evidence for an enolpyruvate intermediate can be cited for the first two of these on the basis of their stereospecific ketonization of added enolpyruvate (Kuo & Rose, 1982). In addition, enolpyruvate has been inferred as the intermediate and side product in the PEP carboxy transphosphorylase reaction on the basis of the nonstereospecificity of pyruvate formed in the absence of CO_2 (Willard & Rose, 1972).

Enzyme-bound enolpyruvate measurements (Figure 2) were corrected for a small but quantifiable background of enolpyruvate in the reaction mixture without pyruvate kinase. The background was found to be linear in pyruvate concentration (extrapolating to zero in the absence of phosphoenolpyruvate), was found to be slightly dependent on pH, and was more dependent on the type of divalent metal ion used (data not shown). In the experiments reported here, the background was less than 15% of the bromopyruvate formed in the presence of millimolar concentrations of pyruvate kinase. Catalytic concentrations of pyruvate kinase were found necessary to lower background levels and introduce good reproducibility. It appeared as if the enzyme was acting on some component arising from the concentrated pyruvate stock solutions used for preparing reaction mixtures.

The apparent solution enol–keto equilibrium constant was severalfold higher than previously determined (Miller & Leussing, 1985; Burgner & Ray, 1974). This apparent discrepancy may be due to additional enolization of pyruvate in the presence of bromine in the acid-quenched samples and/or due to the presence of Mg^{2+} in the present experiments. The former effect is probably small as discussed under Materials and Methods; however, Mg^{2+} forms a complex with enolpyruvate (Miller & Leussing, 1975) which pulls the "apparent" enol–keto equilibrium toward enolpyruvate.

The decrease in affinity of E·K^+·Mg^{2+}·AMPPCP for pyruvate at elevated pHs (Table I, Figure 3) differs from a finding by Lodato and Reed (personal communication), who observed an increase in affinity of pyruvate kinase for pyruvate as pH is increased. These investigators also find inhibition of pyruvate binding by bicarbonate. Since the present experiments were not done in such a way that prevented equilibration of the solutions with atmospheric CO_2, the increased bicarbonate concentrations expected at high pHs may be responsible for the present observation of a decrease in apparent affinity of pyruvate kinase for pyruvate.

Pyruvate Kinase Internal Equilibria. We have found 0.15 equiv of enolpyruvate/pyruvate kinase active site in a system where the full equilibrium was established on the enzyme at pH 9.0 (Table II). Under these same conditions we measured 0.24 equiv of PEP. Since the dissociation constant of PEP is only 0.05 mM and probably less for the ternary substrates complex and we are using 1 mM enzyme, the PEP determined should be almost completely bound. The remainder of the enzyme, 0.61 equiv (i.e., 1 − 0.15 − 0.24), can be expected to be occupied by pyruvate since the pyruvate concentration is more than 5 times greater than its dissociation constant. Hence, the ternary complexes E·ATP·Pyr, E·ATP·enol-Pyr, and E·ADP·PEP exist in the proportions of 0.61:0.15:0.24 to a first approximation. The enolization equilibrium in the complete system with ATP, 0.15/0.61 = 0.24, is in good agreement with the value of 0.22 found with AMPPCP, interpolating to pH 9 (Table II and Figure 3). It should be noted, however, that the present estimations of K_2 and K_3 represent respective upper and lower limits of the actual internal equilibria due to the assumptions made about the fullness of enzyme occupancy. Also in agreement is the ATP/ADP ratio of 3.3 derived from Figure 5 and that calculated from these numbers [(0.61 + 0.15)/0.24 = 3.2]. These values also allow calculation of the internal kinase equilibrium as being 0.15/0.24 = 0.62 under these conditions. K_2



was found to increase ~10-fold in the region pH 8–9 and become pH independent above pH 9. The equilibrium as written may represent the conditions above pH 9. This would satisfy the expectation that the conjugate acid of the catalytic base would be highly constrained from ionizing in the enolate intermediate state (Rose et al., 1990). ^{17}O–Mn EPR studies (Lodato & Reed, 1987) make it clear that the enolate oxygen will be stabilized in the inner coordination sphere of Mn^{2+}, putting the pK_a of the enol out of the pH range studied here. The decrease in K_2 below pH 9 may result from

with a $pK_a \sim 9.1$, possibly appropriate to Lys269 the group designated from X-ray crystallography results as the specific base catalyzing the enolization step (Muirhead et al., 1986). Dougherty and Cleland (1985a,b) have assigned a lysine with a pK_a of ~9.1 in the free enzyme as being the catalytic base responsible for enolization. If these assignments are correct, it is interesting to note that the pK_a of this lysine remains unchanged by pyruvate binding.

As determined by Rao et al. (1979) and Stackhouse et al. (1985), the overall internal equilibrium of the pyruvate kinase reaction, not considering the central intermediate state, is closer to unity than the same reaction in solution. This is verified here, $K_{eq} = 3.3$ compared with 6500. In addition, this can be said of the two-component equilibria. The pH dependence of the concentration of E·ATP·enolpyruvate causes it to be very low at some working pHs of the enzyme in vivo. Therefore, it appears that pyruvate kinase is functionally adequate without closely matched energetic states.

Whatever the origin of the effect of pH on the internal enol–keto equilibrium, its direction may play a major role in determining the pH dependence of the rate of detritiation of bound [^{3}H]pyruvate (Rose, 1960; Robinson & Rose, 1974). An excellent direct correlation now exists between the position of the enol–keto equilibrium, K_2, and the rate of detritiation of bound pyruvate, not only for experiments with Mg^{2+} as reported here but also for experiments with Co^{2+} which have a biphasic pH profile in K_2, peaking at pH ~7.5 (data not shown), in close parallel to the pH profile of the pyruvate kinase catalyzed detritiation rate of [^{3}H]pyruvate with Co^{2+} as the divalent metal ion cofactor. Such a good correlation may suggest that the rate-limiting step for detritition of pyruvate might be in ammonium rotation (positional isotope exchange) if lysine is the catalytic base or in internal chemical exchange if the conjugate acid of the base is monoprotonic.

Registry No. Pyruvate kinase, 9001-59-6; enolpyruvic acid, 19071-34-2.

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