was added 60 mg of 10% Pd on charcoal. The mixture was hydrogenated at room temperature and pressure overnight and absorbed 15.8 mL (approximately 1 equiv) of hydrogen. The catalyst was filtered off and the filtrate evaporated to dryness. Attempts to recrystallize it were unsuccessful: mp 113-115 °C, 93-96 °C phase change; $[\alpha]_D$ +53° (c 3.59); UV (methanol) λ_{max} 288 nm (ϵ 209); IR 3450, 1750, 1720, and 1630 cm^{-1}; NMR δ 4.93 (AB quartet, 2, J = 18 Hz, $\Delta\nu$ = 50 Hz, H-21), 4.06 (s, 1, H-1), 3.25 (s, 3, OCH_3), 2.14 (s, 3, acetate), 1.61 (s, 3, H-19), and 0.81 (s, 3, H-18).

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Physical, Chemical, and Enzymological Characterization of Enolpyruvate[1]

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Abstract: Acid and alkaline phosphatase reacts with phosphoenolpyruvate (PEP) to generate enolpyruvate, but neither ketopyruvate nor the geminate diol of pyruvate. The adsorption spectrum for the phosphatase product was derived by kinetic correlation of the changing spectra. Its λ_{max} (225 nm) and molar absorptivity, ϵ_{225} 9600 M^{-1} cm^{-1}, are appropriate for 2-hydroxyacrylic acid. Mass spectral analysis shows that the phosphatase product that accumulates transiently requires the addition of a proton to C-3 to give pyruvate. The protonation is slowed ca. sixfold in D_2O compared to H_2O. Enolpyruvate has sufficient stability ($t_{1/2}$ = 3.6 min at 20 °C in D_2O, pD 6.4) to be examined as a possible intermediate in enzymatic catalysis. It was predictably shown to lead to strong inhibition of lactate dehydrogenase in the presence of DPN^+. Catalysis by pyruvate kinase of the ketonization of enolpyruvate, generated with phosphatase in situ, was shown to occur with $k_{cat.}$ = 50 min^{-1} and to require both K^+ and Mg^{2+}. The apparent K_M is $\sim 10^{-8}$ M. The rate of ketonization is about 11% of the rate of the overall reaction: ADP + PEP → ATP + pyruvate.

Introduction

The enolic form of pyruvate may be considered a possible transient intermediate in many enzyme-catalyzed reactions specific for phosphoenolpyruvate (addition at C-3 or replacement of the $-PO_3$ group) and pyruvate (substitution at C-3).[2] We have recently reported[3] that the product of phosphatase action on phosphoenolpyruvate (PEP), presumed to be enolpyruvate, is converted stereospecifically to pyruvate by muscle pyruvate kinase. In the present paper we provide evidence of the physical and chemical nature of the phosphatase product that characterizes it as enolpyruvate. In addition, further studies with pyruvate kinase are reported indicating the kinetic properties and cofactor requirements for the ketonization.

Experimental Section

Materials. Phosphoenolpyruvate (tricyclohexylamine salt) and L-lactate dehydrogenase (LDH, EC 1.1.1.27) from beef heart were purchased from Sigma Chemical Co. and D-lactate dehydrogenase (EC 1.1.1.28) of *Lactobacillus leichmannii* was from Boehringer. When PEP was used for spectral studies, it was passed through Dowex AG 50W-X4 (H^+ form, Bio-Rad) and Chelex-100 to remove cyclohexylammonium cation and contaminating metal ions. Acid phosphatase from potatoes (type IV of Sigma or grade I of Boehringer) and alkaline phosphatase from calf intestine (type VII of Sigma) or *Escherichia coli* (type IIIS of Sigma) could be used interchangeably as a source of enolpyruvate at pD 6. Pyruvate kinase (EC 2.7.1.40) from rabbit muscle was obtained from Boehringer-Mannheim Biochemicals. The ammonium sulfate suspension of each enzyme was centrifuged at 15000*g* for 10 min, taken up in 99.8% D_2O (Aldrich

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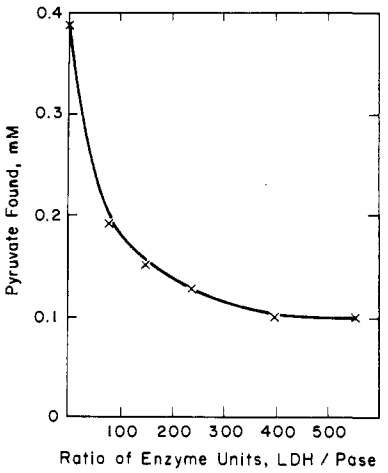


Figure 1. Evidence for an intermediate between PEP and pyruvate formed by phosphatase. Each sample contained in 0.2 mL, 250 μg (0.075 unit of Sigma IV) of acid phosphatase (Pase), 1 μmol of DPNH, 10 μM sodium maleate (pH 6.0), and 0, 6, 12, 18, 30, or 42 units of LDH. PEP, 1 μM, was added to the reaction at 20 °C and after 1 min 0.05 mL of 12 N $HClO_4$ was added. After 10 min at room temperature, 0.3 mL of 2 N $KHCO_3$ was added to neutralize the solution. Pyruvate was determined after centrifugation.

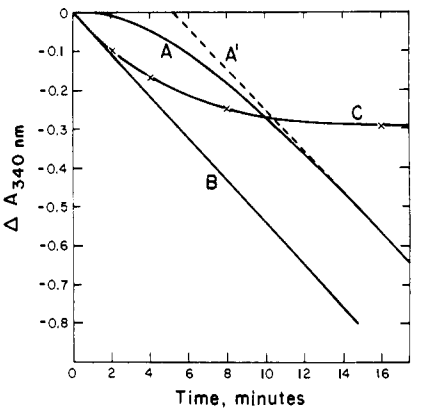


Figure 2. Kinetic evidence for the intermediate. The incubation contained 10 μmol of PEP, 1.25 μmol of DPNH, 44 units of LDH, 0.044 unit of acid phosphatase (Sigma IV), and 20 μmol of maleate at pD 6.40, 20 °C, in 5 mL of D_2O. The reaction was initiated with PEP and followed by A_{340} decrease (curve A). Aliquots of 1 mL were quenched with 0.1 mL of 12 N $HClO_4$, at 2, 4, 8, and 16 min. After 10 min at room temperature, 0.6 mL of 2 N $KHCO_3$ was added to neutralize the solution. Pyruvate was determined after centrifugation. Curve B is the steady-state rate. Curve C is derived from the absorbance of curve B minus curve A at that time. The four points on curve C represent the A_{340} of pyruvate found after quenching.

Scheme I

$$\text{PEP} \xrightarrow{V} \text{enolpyruvate} \xrightarrow{k + k_{enz}} \text{pyruvate}$$

$$\xrightarrow{k_3\ (\text{LDH})} \text{lactate}$$
$$\text{DPNH} \quad \text{DPN}^+$$

Chemical Co., distilled), and passed through columns of Sephadex G-25 equilibrated with D_2O to remove ammonium sulfate and H_2O.

Methods. Generation of Enolpyruvate. The method employed depends on the action of a phosphatase on PEP. LDH and DPNH are present to give a continuous assay of pyruvate formation by ΔA_{340nm} (Scheme I). Because enolpyruvate is not a substrate for LDH, the oxidation of DPNH will show a lag, the length of which depends on the rate of ketonization of enolpyruvate.

Each step of the sequence will be essentially irreversible in view of the very slow rate of enolization of pyruvate,[4] $\sim 4 \times 10^{-6}$ s^{-1}, and the position of the LDH equilibrium ($K_{eq} \simeq 3 \times 10^5$, pH 6.0).[5] By analogy with a similar problem treated by Wurster and Hess,[6] concentrations of enolpyruvate and DPN^+ at any time, t, are:

$$[\text{enolpyruvate}]_t = V\tau(1 - e^{-t/\tau}) \quad (1)$$

and

$$[\text{DPN}^+]_t = V[t - \tau + \tau e^{-t/\tau}] \quad (2)$$

respectively. $\tau = [k + k[E]]^{-1}$ is the transient time obtained by extrapolation to the time axis of the DPN-rate curve from the extension of the steady-state line (A'), as in Figure 2. The steady-state enolpyruvate concentration is given by $V\tau$ or by the absolute value of the intercept on the absorbance axis by line A'.

Determination of the UV Spectrum of Enolpyruvate. According to the following scheme:

$$\text{PEP} \xrightarrow{V_m} \text{enolpyruvate} \xrightarrow{k_2} \text{pyruvate}$$

The observed absorbance at one wavelength is a function of time as described by eq 3:

$$A_{obsd} = A_{PEP} + V_m t(\epsilon_{py} - \epsilon_{PEP}) + (\epsilon_{enolpy} - \epsilon_{py})(1 - e^{-k_2 t})\frac{V_m}{k_2} \quad (3)$$

where ϵ_{py}, ϵ_{PEP}, and ϵ_{enolpy} are molar absorptivities of pyruvate, PEP, and enolpyruvate, respectively, at the specific wavelengths measured.

One takes the time derivative of eq 4:

$$\frac{dA_{obsd}}{dt} = V_m(\epsilon_{py} - \epsilon_{PEP}) + (\epsilon_{enolpy} - \epsilon_{py})V_m e^{-k_2 t} \quad (4)$$

The rate of change in absorbance becomes invariant (dA_{obsd}/dt) = S, when the concentration of enolpyruvate becomes steady; $S = V_m(\epsilon_{py} - \epsilon_{PEP})$. It follows that:

$$\ln\left(\frac{dA_{obsd}}{dt} - S\right) = -k_2 t + \ln[(\epsilon_{enolpy} - \epsilon_{py})V_m]$$

From a plot of $\ln[(dA_{obsd}/dt) - S]$ vs. time, the extrapolation to $t = 0$ gives $I = \ln[(\epsilon_{enolpy} - \epsilon_{py})V_m]$. Then:

$$\epsilon_{enolpy} = \epsilon_{py} + (\epsilon_{py} - \epsilon_{PEP})\frac{e^I}{S} \quad (5)$$

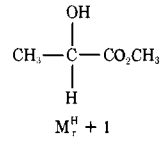
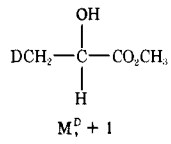
With the known values of ϵ_{py} and ϵ_{PEP}, eq 5, the initial and steady-state rates of absorbance change are used to find $\epsilon_{enolpyruvate}$ at each wavelength.

Mass Spectral Analysis. Methyl lactate was analyzed by gas chromatography-mass spectrometry on a Finnigan Model 3300 instrument (Columbia University, Department of Chemistry). A 6-ft capillary column containing 4% silicone rubber OV-1 (80-100 WHP 57111) was used and methane was used as a chemical ionization source. The signal intensities at m/e 105, 106 were recorded.

Results

Proof of Formation of Enolpyruvate. PEP was treated with phosphatase in the presence of LDH and DPNH to assay pyruvate as it was produced. The reaction was allowed to proceed as described in Figure 1 for 1 min, at which time it was acidified to inactivate both enzymes. The neutralized solutions of reaction products were assyed for pyruvate with fresh LDH/DPNH. The presence of pyruvate even at very high LDH/phosphatase ratios was taken as evidence for the existence of enolpyruvate at the time of acidification or some other compound capable of forming pyruvate during the acidification and neutralization. The amount of pyruvate found in the limit of LDH/phosphatase of ~250 was 0.1 mM, which is ~1000× more than expected given the 42 units of beef heart LDH with its K_m of 0.14 mM for pyruvate.[7] The finding that at 1 min, which is pre-steady state, only 25% of the hydrolyzed PEP is present as an intermediate indicates that the intermediate is rapidly ketonized, $t_{1/2} < 0.5$ min. In control experiments, in which the acid and PEP were added together to solutions of both enzymes with DPNH, no pyruvate was detected.

Table I. Methane Chemical Ionization Mass Spectrometer Analysis to Identify Enolpyruvate

species	mass no.	magnitude	$\left(\frac{[^1H]pyruv}{[^2H]pyruv}\right)_{obsd}$ by mass ratio[a]	$\left(\frac{enolpyruv}{pyruv}\right)_{calcd}$ by rate expressn[b]
 $M_r^H + 1$	105	100	1.22	1.17
 $M_r^D + 1$	106	82.7		

[a] The incubation contained 10 μmol of PEP, 7.5 mg of acid phosphatase, and 50 μmol of sodium citrate at pD 4.9 in 1 mL of D_2O at 15 °C, and the reaction was allowed for 6 min. It was quenched with 15 mL of 2 N $HClO_4$ at room temperature for 30 s and then neutralized with 15 mL of 2 N $KHCO_3$. Pyruvate was converted to lactate by lactate dehydrogenase. The lactate was extracted overnight by continuous extraction with ether. It was methylated by diazomethane and analyzed by gas chromatography-mass spectrometry. [b] Calculation based on eq 1.

Table II. Comparison of D_2O Isotope Effect on Ketonization of Enolpyruvate by UV Method and Mass Spectrometer

species	mass no.	magnitude	k_{H_2O}/k_{D_2O} after ^{13}C correction[a]	k_{H_2O}/k_{D_2O} rate measurement[b]
$M_r^H + 1$	105	68.1		
$M_r^D + 1$	106	100	6.2	6.0

[a] The reaction contained 10 μmol of PEP, 4 mg of acid phosphatase, 10% H_2O, 90% D_2O, 50 μmol of citrate at pH 4.5, 15 °C, and was quenched with $HClO_4$ and neutralized after the reaction was completed. The pyruvate was reduced by LDH, and the lactate extracted and analyzed as in Table I. The procedure described in Table I was used for mass analysis of methyl lactate. [b] The rate ratio was derived from the transient time according to the method in Figure 2, using the conditions given above with either 100% H_2O or D_2O.

Formation of the intermediate between PEP and pyruvate was followed by measuring the time course of DPNH oxidation at high LDH (Figure 2). Assuming that the intermediate is enolpyruvate, these and subsequent kinetic experiments were done in D_2O, which would slow the rate of ketonization. The lag in the production of pyruvate, line A, can be characterized by the transient time, τ = 5.3 min, which is obtained by extrapolating the steady rate to the time axis, line A'. The steady rate measured in this way would also be the rate of the phosphatase through the whole time course as long as the change in PEP concentration was small, as in this study. The phosphatase rate is then represented by line B, drawn parallel to A' through the origin. When the DPNH consumed at each time is subtracted from total PEP hydrolyzed, curve C is generated, which would represent the concentration of the intermediate(s) present at each time. Samples were analyzed as in Figure 1 for pyruvate formed after acidification prior to and in the steady-state region. The values obtained are seen to fall on line C, Figure 2. Therefore, the lag in production of pyruvate from PEP is *completely* accounted for by the accumulation of a compound that produces pyruvate upon acidification but is not pyruvate at the time the reaction is stopped with acid. The steady concentration of enolpyruvate was correctly predicted from $V\tau$ from eq 1, $t > \tau$. Because LDH has broad specificity for α-keto acids, we suppose that the process measured by the lag is the ketonization of the intermediate with a rate constant, τ^{-1} = 0.19 min^{-1} at pD 6.4, 20 °C, in D_2O.

The first-order rate constant calculated from the steady-state rate and the determined concentration of enolpyruvate were not altered over the range of phosphatase activities from 4 to 40 mmol/min and with LDH activities of 1.6-16 units/mL. Acid phosphatase from potato and alkaline phosphatase from *E. coli* gave similar results. As reported earlier,[3] the pyruvate formed from reaction of the phosphatase with (*E*)-[3-^{3}H]PEP in D_2O was achiral 3(H,^{2}H,^{3}H)pyruvate. Therefore, ketonization probably occurred in solution from liberated enolpyruvate.

Two possibilities were suggested for the nature of the intermediate: enolpyruvate and the geminate diol form of pyruvate, neither of which is expected to be a substrate for LDH.[8] The latter seems unlikely to have formed without first forming free pyruvate unless it was liberated from the phosphatase as such. It was therefore desirable to show that the intermediate present at the time of acid quenching did not have a C-3 methyl group. To do this, the reaction mixture in D_2O was terminated by acid-H_2O at a time when the products of the phosphatase reaction were about equally pyruvate and intermediate. Pyruvate formed from enolpyruvate after termination would contain no deuterium. The results of mass spectral analysis show that none of the intermediate calculated to be present at the time the phosphatase reaction was terminated contained a deuterium atom (Table I).

When phosphatase action on PEP was conducted in H_2O/D_2O = 0.11, the ratio of CH_3/CH_2D pyruvate formed was 0.68, corresponding to an isotope discrimination of 6.2 (Table II). The results indicate that proton transfer occurs in the rate-determining step of pyruvate formation consistent with the accumulation of an intermediate that has not yet ketonized.

Ultraviolet Spectrum of Enolpyruvate. The absorbance at several wavelengths was followed during phosphatase action on PEP (Figure 3), in order to establish the spectrum of enolpyruvate shown in Figure 4. The molar extinction coefficient at the λ_{max} (ϵ_{225} 9600 M^{-1} cm^{-1}) compares with an ϵ_{195} of 10 000 for acrylate.[9] An α-hydroxyl group is expected to cause a red shift of ~30-35 nm, which would account for the λ_{max} of 225 observed.[9] The ultraviolet spectrum also demonstrates that neither ketopyruvate nor the geminate diol of pyruvate is the intermediate being investigated.

Inactivation of Lactate Dehydrogenase by Phosphatase Product. It is well established that DPN plus pyruvate brings about the slow loss of LDH activity.[10] This effect is believed to result from the formation on the enzyme of a covalent adduct between DPN^+ and enolpyruvate that is present in a solution of pyruvate.[4,11] Consistent with these observations, it was found that when DPN^+ is present initially in the usual coupled phosphatase-LDH assay the LDH becomes inhibited rapidly (Figure 5). Addition of pyruvate at 7 min gave no increase in the rate of oxidation of DPNH, indicating that pyruvate itself

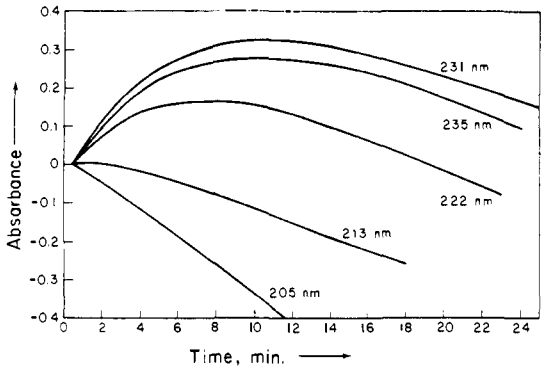


Figure 3. Absorbance changes during enolpyruvate formation at several wavelengths. Each sample containing 20 mM acetate, acid phosphatase (0.1 unit, Boehringer), and 0.5 mM PEP in 0.5 mL of D_2O at 15 °C, pD 5.0, was observed in 0.2-cm path length cuvettes against a blank that contained the same reagents except for PEP. The reaction was initiated with PEP. A Cary 16 double beam spectrophotometer, temperature regulated water bath, and a Honeywell recorder (Electronik 16) were employed.

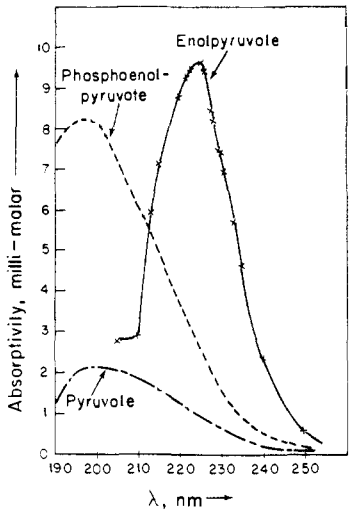


Figure 4. Ultraviolet spectra of enolpyruvate, pyruvate, and PEP. Using the data of Figure 3, including additional wavelengths, the spectrum of enolpyruvate was calculated according to eq 5. Also shown are the spectra of PEP and pyruvate.

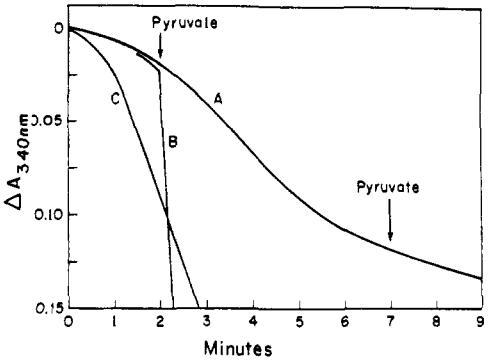


Figure 5. Inactivation of LDH by enolpyruvate and DPN^+. The rate of DPNH oxidation was followed at A_{340nm} in a solution containing in D_2O, 15 °C: sodium maleate (40 mM, pD 6.0), EDTA (0.1 mM), $MgCl_2$ (1 mM), KCl (50 mM), DPNH (0.12 mM), DPN^+ (50 μM), acid phosphatase (Sigma IV, 150 μg), D-LDH (25 μg), and PEP (1 mM) added last. Pyruvate (0.1 mM) was added as noted by (↓). Pyruvate kinase (25 μg) was present in C only.

Table III. Kinetics of Enolpyruvate Ketonization by Pyruvate Kinase[a]

expt	[Pase], μg	[PEP], μM	[enolpyruvate], μM	LDH rate at steady state, nmol min^{-1} mL^{-1}	obsd $(k + k_e)$,[b] min^{-1}
1	150	964	27	6.7	0.25 (= k)
2	150	948	12.2	13.3	1.09
3	150	460	8.4	10.3	1.55
4	300	175	7.5	16.0	2.13
5	300	472	10.6	19.3	1.82
6	600	443	18	37.8	2.10
7	1200	391	29	74.0	2.55

[a] Conditions were those of Figure 5 with PEP and phosphatase varied. PEP and enolpyruvate, as pyruvate, were determined when steady state was approximated, at least 3.5τ. Pyruvate kinase (25 μg) was present in all incubations except expt 1. [b] $(k + k_e)$ = (steady-state LDH rate)/(enolpyruvate).

had accumulated at this time. This result was obtained with both the D-lactate dehydrogenase of bacteria and L-lactate dehydrogenase of beef heart muscle.

Catalysis of Ketonization by Pyruvate Kinase. Previously we have reported that in the presence of muscle pyruvate kinase the ketonization of (*E*)-[3-^{3}H]PEP with phosphatase occurs with almost complete stereospecificity by addition of $^2H^+$ to the *si* face of presumed enolpyruvate.[3] A catalysis of ketonization could also be anticipated to protect LDH from inhibition by DPN^+ plus enolpyruvate, and this is observed (Figure 5) (line C). The lag time is greatly shortened and, although not shown, the unlimited rate of LDH was observed to continue for at least 9 min. The apparent increased rate due to pyruvate kinase did not result from a phosphatase action of this enzyme previously shown[12] to be significant with Co^{2+} and Ni^{2+} but not Mg^{2+}. Mg^{2+} and K^+ were present in incubations of Figure 5 to accommodate the requirements of the kinase, as will be shown later.

The amount of pyruvate kinase required to shorten the lag shown in Figure 5 is quite large compared with the rate of production of pyruvate from PEP + ADP. The latter rate, when assayed at pD 6.0, 15 °C, was only ~4.5% of its rate at pH 8, 25 °C. Therefore, the 25 μg of enzyme present would have had a maximum rate of ATP + pyruvate formation of ~5 μmol/min compared with the steady-state rate of ketonization observed, ~0.01 μmol/min. The question then arises as to what factors limit the rate of enzyme catalysis.

One factor responsible for this is, most certainly, that PEP will act as an inhibitor, presumably competitive, with respect to enolpyruvate, and therefore the enzyme may not be operating maximally at the established steady-state level of enolpyruvate. To evaluate the kinetic parameters for enolpyruvate it was necessary to compensate for the effect of PEP in the usual v^{-1} vs. [enolpyruvate]$^{-1}$ representation. Two approaches gave useful data. In one the PEP level was varied and in the other the phosphatase was varied at a given level of PEP. The results are summarized in Table III. Inhibition by PEP is clearly seen by comparison of k_e and [PEP], where the greatest rate constant is seen at the lowest [PEP]. The usual rate equation for competitive inhibition can be simplified in this case because the concentrations of PEP, in all cases, are much greater than the binding constant, ~10^{-6} M.[13] This leads to the simplification of eq 6:

$$k_e = k_{cat}/1 + \frac{K_M}{[\text{enolpyruvate}]}\left(1 + \frac{[\text{PEP}]}{K_i}\right) \simeq k_{cat}/1 + \frac{K_M}{K_i}\frac{[\text{PEP}]}{[\text{enolpyruvate}]} \quad (6)$$

Table IV. Requirement of K^+ and Mg^{2+} for Ketonization of Enolpyruvate by Pyruvate Kinase[a]

metal cofactors				ketonization rate (PEP + Pase ± 25 μg of P.K.)[a]		phosphoryl transfer rate (PEP + ADP + 0.5 μg of of P.K.)[b]	
expt	$[Mg^{2+}]$, mM	$[K^+]$, mM	P.K.	expt	k_{obsd}, min^{-1}	expt	$-\frac{\Delta A}{\Delta t}$, min^{-1}
a, h	1	50	yes	a	0.422	h	0.1480
b, i	1	0	yes	b	0.241	i	0.0410
c, j	0	50	yes	c	0.131	j	0.0025
d, k	0	0	yes	d	0.109	k	0.0003
e	1	0	no	e	0.139		
f	0	50	no	f	0.128		
g	0	0	no	g	0.097		

[a] P.K., pyruvate kinase. The method was described in the legend of Figure 2. The incubation contained, in 1 mL of D_2O, 15 °C, 40 mM sodium maleate, 0.12 mM DPNH, 2 units of LDH, 25 μg of acid phosphatase, 1 mM PEP, and the additions noted. The reaction was initiated with PEP. Full scale, 0.5 o.d.; chart speed, 1 in./min. The first-order rate constant, $k_{obsd} = \tau^{-1}$, where τ is the transient time. [b] The incubation at 15 °C in 1 mL of D_2O contained 40 mM sodium maleate, 0.5 mM ADP, 0.15 mM DPNH, 2 units of LDH, 1 mM PEP, and the additions noted in the left part of the table. $-\Delta A/\Delta t$ is the rate of absorbance change at 340 nm.

The kinetic constants for reaction of enolpyruvate with pyruvate kinase are obtained by plotting the observed k_e^{-1} against [PEP]/[enolpyruvate] (Figure 6). The linearity of the plot suggests that PEP and enolpyruvate concentrations are the major factors in changing the rate. It was possible to calculate $k_{cat} \simeq 3$ min^{-1} from the intercept on the ordinate and to calculate the ratio (K_i of PEP)/(K_M of enolpyruvate) of 40 from the value at $k_e = 1.5$ min^{-1}.

Requirements for Mg^{2+} and K^+. It has long been known that both monovalent and divalent cations, best represented by K^+ and Mg^{2+}, are required for pyruvate kinase to catalyze the enolization of pyruvate[14] as well as the net overall reaction.[15] Recently, it was shown that the divalent cation requirement for pyruvate kinase includes one Mg^{2+} in addition to MgADP.[16] Table IV examines the requirement for metals of the pyruvate kinase catalyzed ketonization of enolpyruvate. Strong stimulation of the enzymatic ketonization of enolpyruvate is shown by K^+ only in the presence of Mg^{2+} (compare the difference between expts c-d and a-b) and by Mg^{2+} only when K^+ is also present (compare b-d with a-c). The stimulation caused by both metals together is greater than the sum of the separate effects, i.e., (c-d) + (b-d) compared with (a-d), 0.144 vs. 0.313. The activity of the enzyme in the absence of added K^+ (b-d = 0.132) is very probably facilitated by the ~50 mM Na^+ present in the buffer as shown by the ~30% of maximal rate when the overall reaction with PEP was measured; compare h and i.

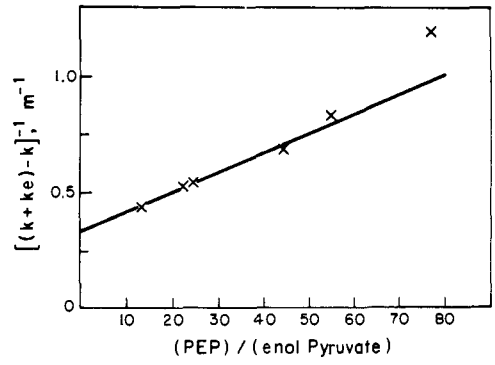


Figure 6. Determination of V_{max} and K_m of enolpyruvate for pyruvate kinase. The observed ketonization rate due to pyruvate kinase is plotted as its reciprocal against [PEP]/[enolpyruvate]; data from Table III. At saturation, $k_e = 3$ min^{-1} and $K_m = K_{i,PEP}/40$.

Discussion

From the data in Table III and from Figure 6 one can calculate the maximum rate of enzyme-catalyzed ketonization as follows: At PEP/enolpyruvate for which the ketonization rate is half-maximal, the rate due to half of the 25 μg of enzyme was about 13 nmol min^{-1} mL^{-1}. This corresponds to a turnover of about 50 min^{-1} per subunit of 50 000 which is 200× the spontaneous rate. This turnover rate constant is about 11% as great as that obtained for the formation of pyruvate from PEP plus ADP under the same reaction conditions. Although this is well below the minimum rate expected for an intermediate, there are several factors that need to be considered. It is not possible to explain more than 10% of the enzymatic rate of ketonization by reaction of the enolate ion present in equilibrium with the enolpyruvate without assuming a second-order diffusion rate constant $>10^8$ M^{-1} s^{-1}, which represents the upper limit found for enzymes,[17] or a pK_a for the enol/enolate ionization <11. Diffusion limitation in the range of concentrations of enolpyruvate used should not have resulted in the finite intercept value for V_{max} seen in Figure 6. Therefore, enolpyruvate must be the major substrate form when provided from solution. Its failure to give the expected V_{max} may result from the following possibilities: Enolpyruvate on the enzyme must ionize to the enolate form prior to ketonization by an amino acid proton donor. The ionization of the enol, not being a normal step of the kinase reaction, may be rate determining in the environment of tight binding, $K_m = 10^{-8}$ M. Addition of MgATP (1 mM MnATP) was found not to increase the rate of ketonization of enolpyruvate by pyruvate kinase at pD 6.0 and 15 °C, which is consistent with the above interpretation in which formation of E·enolpyruvate is rate determining for ketonization. These arguments predict that the enzymatic rate should not show a D_2O effect and may be rather insensitive to pH in the range pH 6-7. These experiments are made difficult by the strong effects of medium and pH on the nonenzymatic rate of ketonization, however.

A second factor to be considered in lowering the enzymatic ketonization rate in comparison with the overall reaction is that the structure of the active enzyme·enolate species may depend on its route of formation. It is known[18] that PEP with ADP undergoes exchange of hydrogen at C-3 with water under conditions of the initial pyruvate kinase reaction, showing that ATP does not depart from the enzyme prior to ketonization. By its binding to the enzyme, ATP may serve to promote the phosphoryl transfer equilibrium and thereby increase the level of enolate complex in a way that cannot be duplicated if phosphoryl transfer is omitted.

References and Notes

(1) This work was supported by U.S. Public Health Service Grants GM-20940, CA-06927, and RR-05539, and by an appropriation from the Commonwealth of Pennsylvania.

(2) (a) Enzymes that catalyze addition at C-3 of PEP are pyruvate kinase (EC 2.7.1.40), pyruvate, orthophosphate dikinase (EC 2.7.9.1), PEP synthase (EC 2.7.1.dd), phosphoenolpyruvate carboxylase (EC 4.1.1.3.1), phosphoenolpyruvate carboxykinases (EC 4.1.1.32, 4.1.1.a), phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38), and phospho-2-keto-3-deoxy-heptonate aldolase (EC 4.1.2.15). (b) Enolpyruvate transferase (EC 2.5.1.7) catalyzes substitution for the PO_3^{2-} group of PEP. (c) Substitution at C-3 H of pyruvate occurs in pyruvate carboxylase (EC 6.4.1.1), methylmalonyl-CoA:pyruvate carboxyltransferase (EC 2.1.3.1), malate dehydrogenases (decarboxylating) (EC 1.1.1.38 and .40), (4S)-4-hydroxy-2-ketoglutarate aldolase (4.1.2.e), and oxaloacetate decarboxylases (4.1.1.3, biotin-dependent or non-biotin-dependent).
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Chromopeptides from *C*-Phycocyanin. Structure and Linkage of a Phycocyanobilin Bound to the β Subunit

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Abstract: The smallest cyanogen bromide fragment derived from the β subunit of *Synechococcus* sp. 6301 *C*-phycocyanin, the blue heptapeptide **2**, has been investigated by 360-MHz ^{1}H NMR spectroscopy. The peptide portion, heptapeptide **3**, was synthesized independently and used in comparative spectroscopic analysis. These studies have led to complete assignment of the structure of the peptide-linked phycocyanobilin and elucidation of the nature of the thioether chromophore–peptide linkage.

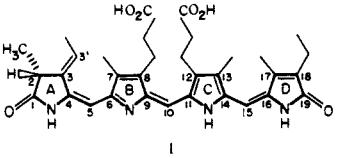
The intensely blue protein, *C*-phycocyanin, is a major light harvesting component of the photosynthetic apparatus of cyanobacteria (blue-green algae) and of certain red algae.[1] Composed of two distinct polypeptide chains, α and β subunits,[2] the monomer of *C*-phycocyanin contains three distinct covalently bound prosthetic groups known as phycocyanobilins, one on the α chain and two on the β chain.[3] Since the initial work in the 1930's,[4] intensive study of the phycocyanobilins has not yet provided the unambiguous assignment of the structure of the natural prosthetic groups or the precise nature of the covalent linkage to the apoprotein.[5,6]

The methodology most widely used to examine the structure of these pigments has entailed the cleavage of the chromophore–protein linkages of *C*-phycocyanin through treatment under various conditions.[7-14] From these studies a number of degradation products and phycobilins released from the protein have been characterized spectroscopically, including the "blue pigment" **1**, whose dimethyl ester has been synthesized recently.[15] It is clear, however, that all of these pigments, while derived from the various native prosthetic groups, are products whose nature is dependent on the cleavage conditions[5] and the potential of the latter for introducing artifacts.

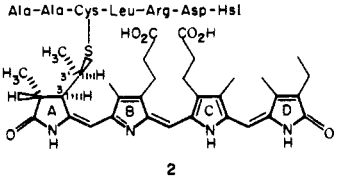
The second experimental approach, which requires the chromophore–protein linkage to be maintained, has been applied to the determination of the amino acid sequences about the sites of attachment of the three phycocyanobilins.[16-19] From such analyses of proteolytically prepared oligopeptides from *C*-phycocyanin, chromophore–protein linkages involving the side chains of serine,[16] aspartic acid,[17] cysteine,[17-19] and tyrosine[19] have been proposed. Two recent studies of highly purified chromopeptides from the *C*-phycocyanins of *Mastigocladus laminosus*[18] and *Synechococcus* sp. 6301,[20] however, have unambiguously established the linkage of a cysteine residue to each of the three phycocyanobilins. Although substantial evidence implicates a thioether linkage,[17,18,20-22] direct proof of the structure of any proposed chromoprotein linkage in *C*-phycocyanin is lacking.

In the present study, we report the structure determination of one of the three peptide-bound phycocyanobilins, namely β_1-phycocyanobiliheptapeptide **2** obtained from the cyanogen bromide cleavage of *Synechococcus* sp. 6301 *C*-phycocyanin.[20,23] A comparison of the ^{1}H NMR spectra of this chromopeptide **2** with those of synthetic peptide **3** has permitted the direct assignment of the structure of the β_1-phycocyanobilin as well as that of the chromoprotein linkage.

Chromopeptide **2**, derived from residues 79–85 of the β chain,[20] was chosen as the substrate to demonstrate a new methodology for the analysis of the prosthetic groups of bil-



1



2

BocNH-Ala-Ala-Cys(SSEt)-Leu-Arg(Mbs)-Asp(Bn)-Hsl

4

↓ HF/EtSH

Ala-Ala-Cys(SSEt)-Leu-Arg-Asp-Hsl

3

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