Lorimer, G. H., Andrews, T. J., & Tolbert, N. E. (1973) *Biochemistry 12*, 18.
McCurry, S. D., & Tolbert, N. E. (1977) *J. Biol. Chem. 252*, 8344–8346.
Mildvan, A. S., & Cohn, M. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 1–69.
Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 2743–2750.
Miziorko, H. M., & Sealy, R. C. (1984) *Biochemistry 23*, 479–485.
Mulligan, R. M., & Tolbert, N. E. (1983) *Arch. Biochem. Biophys. 225*, 610–620.
Pierce, J., & Gutteridge, S. (1985) *Appl. Environ. Microbiol. 49*, 1094–1100.
Pierce, J., & Reddy, G. S. (1986) *Arch. Biochem. Biophys.* (in press).
Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry 19*, 934–942.
Roeske, C. A., & O'Leary, M. H. (1984) *Biochemistry 23*, 6275–6284.
Rose, I. A. (1980) *Methods Enzymol. 64*, 47–59.
Ryan, F. J., & Tolbert, N. E. (1975) *J. Biol. Chem. 250*, 4229–4233.
Saver, B. G., & Knowles, J. R. (1982) *Biochemistry 21*, 5398–5403.
Schloss, J. V., & Lorimer, G. H. (1982) *J. Biol. Chem. 257*, 4691–4694.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 523–533, Wiley, New York.
Somerville, C. R., & Somerville, S. C. (1984) *Mol. Gen. Genet. 193*, 214–219.
Sue, J. M., & Knowles, J. R. (1982) *Biochemistry 21*, 5410–5414.
Weissbach, A., Horecker, B. L., & Hurwitz, J. (1956) *J. Biol. Chem. 218*, 795–810.

Isolation of Pyrophosphohistidine from Pyrophosphorylated Pyruvate,Phosphate Dikinase†

Nelson F. B. Phillips and Harland G. Wood*
Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106
Received August 19, 1985

ABSTRACT: The pyrophosphoryl form of pyruvate,phosphate dikinase was prepared by incubation with adenosine 5′-[γ-^{32}P]triphosphate and isolated by gel chromatography. Previously a phosphorylated moiety had been isolated from the enzyme and was shown to be bound through a phosphoramidate linkage to the 3′ nitrogen of a histidine residue [Spronk, A. M., Yoshida, H., & Wood, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4415]. This histidine residue has been considered to be the pyrophosphoryl and phosphoryl carrier between the three subsites of this enzyme. Previous attempts to isolate the putative [^{32}P]pyrophosphohistidine have been unsuccessful due to the lability of the [^{32}P]pyrophosphoryl-enzyme. By stabilization of the [^{32}P]pyrophosphoryl-enzyme with diazomethane, it has been possible to isolate a [^{32}P]-pyrophosphohistidine from the hydrolysates. To our knowledge this work constitutes the first direct demonstration of a pyrophosphorylated histidyl residue in an enzyme.

Pyruvate,phosphate dikinase (EC 2.7.9.1, pyruvate,orthophosphate dikinase) catalyzes the reversible formation of phosphoenolpyruvate from pyruvate as shown in eq 1.

$$\text{ATP} + \text{P}_i + \text{pyruvate} \rightleftharpoons \text{AMP} + \text{PP}_i + \text{P-enolpyruvate} \quad (1)$$

The overall reaction involves the transfer of the γ-phosphate of ATP to orthophosphate, forming pyrophosphate, and the β-phosphate of the same ATP to pyruvate to produce P-enolpyruvate. The mechanism of these transfers appears to differ depending on the source of the enzyme. Evans and Wood (1968, 1971), using the dikinase isolated from *Propionibacterium shermanii*, proposed a mechanism involving three partial reactions, each of which is catalyzed at a distinct subsite:

$$\text{enzyme} + \text{ATP} \rightleftharpoons \text{E}\sim\text{P}_\beta\text{P}_\gamma + \text{AMP} \quad (1a)$$

$$\text{E}\sim\text{P}_\beta\text{P}_\gamma + \text{P}_i \rightleftharpoons \text{E}\sim\text{P}_\beta + \text{P}_\gamma\text{P}_i \quad (1b)$$

$$\text{E}\sim\text{P}_\beta + \text{pyruvate} \rightleftharpoons \text{enzyme} + \text{P}_\beta\text{-enolpyruvate} \quad (1c)$$

Studies of the enzyme from *P. shermanii* (Evans & Wood, 1968; Milner & Wood, 1972) and from *Bacteriodes symbiosus* (Milner et al., 1978) have shown that both a phosphoryl-enzyme (E∼P)[1] and a pyrophosphoryl-enzyme intermediate (E∼PP) are formed during the reaction. Additional evidence in support of the above tri-uni-uni ping-pong mechanism has come from equilibrium exchange studies (Evans & Wood, 1968; Milner & Wood, 1972, 1976), initial velocity data (Milner & Wood, 1972, 1976), and product inhibition patterns (Milner et al., 1978). The phosphoryl moiety of the E∼P intermediate was shown to be bound to the enzyme through an acid-labile phosphoramidate linkage, and subsequently, $N^{3'}$-[^{32}P]phosphohistidine was isolated from alkaline hydro-

† Supported by PCM 80-22701 from the National Science Foundation.

[1] Abbreviations: E∼P, phosphoryl pyruvate,phosphate dikinase; E∼PP, pyrophosphoryl derivative of the enzyme; P-enolpyruvate, phosphoenolpyruvate; Diazald, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; TFA, trifluoroacetic acid; TEA, triethylamine; ODS, octadecylsilane; PITC, phenyl isothiocyanate; PTC-amino acid, phenylthiocarbamoyl derivative of an amino acid; HPLC, high-performance liquid chromatography; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; NADH, reduced nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-1644$01.50/0 © 1986 American Chemical Society

lysates of [^{32}P]phosphoryl-enzyme (Spronk et al., 1976). The amino acid sequence around the phosphohistidine site has been determined with the enzyme from *B. symbiosus* (Goss et al., 1980). The essential histidyl residue is considered to be centrally located in the enzyme active site and mediates the transfer of the phosphoryl and pyrophosphoryl moieties between the three partial reaction subsites. However, the E~PP form of the enzyme is relatively unstable, and attempts to isolate a pyrophosphorylhistidyl moiety have not been successful.

In contrast to the Evans–Wood mechanism, the pyruvate,-phosphate dikinase isolated from plants involves only two partial reactions and does not imply a pyrophosphoryl-enzyme intermediate (Andrews & Hatch, 1969):

$$\text{enzyme} + \text{ATP} + P_i \rightleftharpoons E\sim P_\beta + \text{AMP} + PP_i \quad (2a)$$

$$E\sim P_\beta + \text{pyruvate} \rightleftharpoons \text{enzyme} + P_\beta\text{-enolpyruvate} \quad (2b)$$

Evidence for this bi-bi-uni-uni mechanism is based mainly on data from partial exchange reaction studies.

Recent kinetic studies (Ciskanik & Dunaway-Mariano, 1983) with the enzyme from *B. symbiosus* have yielded results that are in accord with a bi-bi-uni-uni mechanism rather than the tri-uni-uni mechanism proposed earlier. However, the authors concluded that the chemical mechanism involves both E~P and E~PP. In a later investigation, using positional isotope exchange studies, these authors excluded the formation of E~PP (Wang et al., 1984).

In view of these reports, we decided to see if we could isolate the putative histidyl pyrophosphate by trapping and stabilizing the [^{32}P]pyrophosphoryl-enzyme with diazomethane prior to its isolation. This approach had been used successfully to trap and demonstrate a carboxyl phosphate intermediate as its trimethyl derivative in the reaction catalyzed by glutamate-dependent carbamoyl phosphate synthetase (Powers & Meister, 1976).

Experimental Procedures

Materials

[γ-^{32}P]ATP was obtained from New England Nuclear; phosphoenolpyruvate and NADH were from P-L Biochemicals; pyruvate, nucleotides, hydroxylamine hydrochloride, iodoacetic acid, pepsin, and leucine aminopeptidase were from Sigma Chemical Co.; pyridine was from MCB Reagents; *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) and triethylamine were from Aldrich Chemical Co.; acetonitrile (HPLC grade) was from EM Science; phenyl isothiocyanate, trifluoroacetic acid, amino acid standard H, and 6 N HCl were from Pierce Chemical Co.; *N*-tosyl-L-phenylalanine chloromethyl ketone–trypsin and α-chymotrypsin were from Worthington Biochemical Corp.; Pronase was from Calbiochem; lactate dehydrogenase was from Boehringer Mannheim. Pyridine and triethylamine were redistilled and stored under nitrogen at −15 °C. All other chemicals were of reagent grade or better.

Methods

Purification and Assay of Pyruvate,Phosphate Dikinase. Pyruvate,phosphate dikinase was prepared from *Propionibacterium shermanii* grown on lactate medium by a method similar to that described by Goss et al. (1980). The enzyme was assayed by measuring the rate of pyruvate formation from P-enolpyruvate by coupling with lactate dehydrogenase.

Preparation and Purification of Reagents. (A) Diazomethane. Diazomethane was prepared by the procedure described by Vogel (1967). In a typical experiment, 2.14 g of Diazald was dissolved in 30 mL of ice-cold diethyl ether, and 10 mL of alcoholic KOH (0.4 g of KOH in 10 mL of 96% ethanol) was added. More ethanol was added to dissolve the precipitate. After 5 min at 4 °C, the ethereal diazomethane was distilled off at 60 °C and collected in a smooth glass flask cooled on ice.

(B) [γ-^{32}P]ATP. The crude [γ-^{32}P]ATP, which had 10% contaminating $^{32}P_i$, was purified by the method of Symons (1977) to 99% homogeneity as judged by thin-layer chromatography on poly(ethylenimine)–cellulose plates developed in 0.8 M NH_4HCO_3.

(C) Formation of Pyrophosphoryl-Enzyme. The pyrophosphoryl-enzyme was prepared by reaction with [γ-^{32}P]ATP followed by chromatography on a Sephadex G-50 column as described by Milner et al. (1978). For stability determination of E~PP, 5.6 nmol of the enzyme (specific activity = 11) was incubated in 50 mM Tris–acetate buffer (pH 7) containing 2 mM $MgCl_2$, 10 mM NH_4Cl, and 0.65 mM [γ-^{32}P]ATP (specific activity 1.05×10^5 cpm/nmol) in a final volume of 300 μL. After 10 min at room temperature (20 °C), the E~P^{32}P was rapidly desalted by the microcentrifuge desalting technique as described by Helmerhorst and Stokes (1980). A 3-mL syringe containing Sephadex G-25 (superfine) preequilibrated with 50 mM Tris–acetate (pH 7) containing 0.2 mM EDTA, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, and 0.1% bovine serum albumin was used for this purpose. By this method about 88% of the initial enzyme was recovered as E~P^{32}P. In a parallel experiment, when bovine serum albumin was substituted for the enzyme, the contamination with [γ-^{32}P]ATP was less than 1%.

Amino Acid Analysis. Dried samples were dissolved in 50 μL of 6 N HCl containing 0.1% mercaptoethanol and transferred to micro borosilicate glass tubes. The tubes were vacuum-sealed, and hydrolysis was at 110 °C for 24 h. The hydrolysate was evaporated by rotary evaporation and redried from 100 μL of coupling buffer (acetonitrile/pyridine/TEA/H_2O, 10:5:2:3) along with 100 pmol of norleucine.

(A) Coupling of the Hydrolysate with PITC. The method employed was an adaptation of the procedures described by Heinrikson and Meredith (1984) and Tarr (1982). To the dried hydrolysate was added 50 μL of "aged" PITC (90% ethanol/TEA/PITC, 70:20:10; kept at 20 °C for 10 min), and the mixture was reacted under N_2 at room temperature for 15 min. The solution was then dried by rotary evaporation under high vacuum (vacuum pump $\simeq$100 mtorr). The residue was dissolved in 20 μL of 0.1 M ammonium acetate (pH 6.45) (buffer A) and analyzed by reverse-phase HPLC as described below.

(B) Amino Acid Standard. To prepare the PTC-amino acid standard, 20 nmol (except Cys, which was 10 nmol) of the Pierce standard mixture of amino acids (type H) in 0.1 N HCl containing the 17 amino acids Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, and Lys was dried in a test tube (6 × 50 mm), along with 10 nmol of norleucine. To this mixture was added 50 μL of coupling buffer, and the solution was dried by rotary evaporation. The amino acids were coupled with PITC as described above. The individual amino acids listed above, including norleucine, were derivatized separately in order to establish identities of peaks resolved by HPLC.

(C) Separation of PTC-Amino Acids. Separations were performed with the Waters HPLC system consisting of two M6000A pumps controlled by an M680 gradient controller, an M440 fixed wavelength UV detector, and a Spectra-Physics data module integrater. The column was a reverse-phse C_{18}

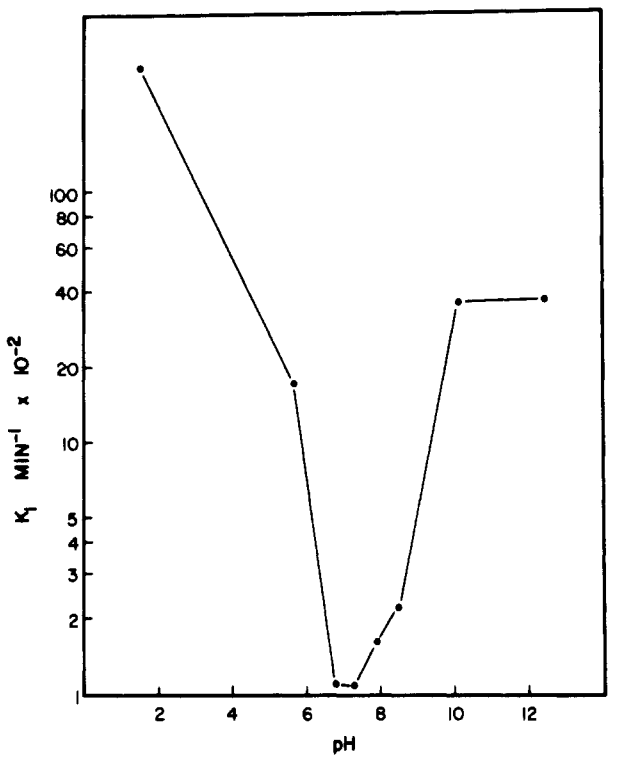

FIGURE 1: Stability of [^{32}P]pyrophosphoryl pyruvate,phosphate dikinase with variation in pH. The first-order decay constant, k_1, is plotted against pH on a semilog plot. The [^{32}P]pyrophosphoryl-enzyme was incubated at 20 °C in 0.1 N HCl (pH 1.5) and the following 0.1 M buffers: Tris–acetate, pH 5.7, 6.8, 7.2, 7.85, and 8.5; $NaHCO_3$, pH 10.1; NaOH, pH 12.5. At different time intervals, aliquots (100 μL) were desalted by the microcentrifuge method and the radioactivity bound to protein was determined. The incubations were for four different periods of time, the times depending on the rates of hydrolysis. For buffers with pH 1.5, 5.7, 10.1, and 12.5, the times were 2, 5, 10, and 30 min, while for buffers with pH 6.8, 7.25, 7.85, and 8.5, the times were 10, 30, 58, and 88 min. The first-order decay constant k_1 was calculated from the slope of the line on semilog graphs in which the percent of [^{32}P]pyrophosphoryl-enzyme remaining was plotted as a function of time ($k_1 = 0.693/t \times 0.5$). A least-squares linear regression analysis was used to calculate the half-lives. For the control, the amount of radioactivity bound to the enzyme at zero time was determined in a neutral buffer.

ODS Ultrasphere from Altex/Beckman (250 × 4.6 mm, 5 μm). The column was jacketed and placed in a water bath maintained at 52 °C. The mobile phase system consisted of two eluants: Buffer A was 0.1 M ammonium acetate (pH 6.45), and buffer B was 60% aqueous acetonitrile. The gradient was run from 10% to 60% buffer B at a flow rate of 1 mL/min for 13 min using a convex curve (curve 5 on M680). The column was washed with 100% buffer B for 5 min before returning to initial conditions.

RESULTS

pH Stability of the Pyrophosphoryl Linkage. In order to evaluate the conditions to be used for the hydrolysis of the pyrophosphoryl-enzyme and isolation of the pyrophospho-amino acid, the stability of the E~PP at different pH values was determined. The first-order rate constants of hydrolysis (k_1) of the pyrophospho-enzyme at different pH values are shown in Figure 1. It is evident that the linkage is very labile at the extremes of pH, imposing a V shape on the curve. The relative stability at the narrow pH range of 6.8 and 7.2 in terms of the half-lives is only 62–64 min. The half-life at pH 10.1 is 2 min at 20 °C compared to 60 days at 37 °C for the phospho-enzyme intermediate (Spronk et al., 1976). The V-shaped curve resembles the hydrolytic curve for acyl phosphates rather than the postulated phosphoramidate linkage.

Table I: Effect of Hydroxylamine and Pyridine on [^{32}P]Pyrophosphoryl Pyruvate,Phosphate Dikinase at 20 °C[a]

compound	k_{obsd} or k_1 (min^{-1})	k_2 (mol^{-1} min^{-1})
−catalyst	0.0084	
pyridine	0.016	0.15
hydroxylamine	0.045	0.73

[a] The [^{32}P]pyrophosphoryl-enzyme (0.96 nmol in 80 μL) was incubated in 0.05 M Tris–acetate, pH 7, with 50 mM hydroxylamine or pyridine or without catalyst, in a final volume of 200 μL. At times of 10, 25, 37, 47, and 60 min, 25 μL was removed into 200 μL of water and unbound radioactivity was removed by Sephadex G-25 microcentrifuge gel filtration; residual [^{32}P]pyrophosphoryl-enzyme was determined. The Sephadex was preequilibrated with 50 mM Tris–acetate, pH 7. The observed first-order rate constants, k_1, were determined at 20 °C at an ionic strength of 1.0, maintained with NaCl. Second-order rate constants, k_2, were calculated as described under Results.

Effect of Hydroxylamine and Pyridine on Pyrophosphoryl Pyruvate,Phosphate Dikinase. In view of the above observation, the occurrence of acyl phosphate could not be completely excluded. Therefore, to obtain additional support for the occurrence of a pyrophosphoramidate type of linkage of the E~PP, the hydrolytic effect of hydroxylamine and pyridine on E~PP was studied, the results of which are shown in Table I. The second-order decay constants (k_2) were calculated by using the equation $k_2 = k_{obsd} - k_1/[\text{catalyst}]$, where k_{obsd} is the apparent first-order decay constant of the pyrophosphoryl-enzyme with catalyst (hydroxylamine or pyridine) and k_1, the rate constant without catalyst (calculated as in Figure 1) (Jencks & Gilchrist, 1965; Spronk et al., 1976). The apparent second-order rate constants for the E~PP form of pyruvate,phosphate dikinase were 0.15 and 0.73 mol^{-1} min^{-1} at 20 °C for pyridine and hydroxylamine, respectively. These data resemble the values obtained for the phosphoramidate hydrolysis at 30 °C, which were 0.7 and 0.8 mol^{-1} min^{-1} for pyridine and hydroxylamine, respectively (Jencks & Gilchrist, 1965). Spronk et al. (1976) obtained values of 0.5 and 0.25 mol^{-1} min^{-1}, respectively, for the phosphoryl pyruvate,phosphate dikinase at 30 °C.

Isolation of the Methylated [^{32}P]Pyrophosphohistidyl Residue. The [^{32}P]pyrophosphoryl-enzyme was prepared by incubating pyruvate,phosphate dikinase (30 nmol) with [γ-^{32}P]ATP as described under Methods. After 10 min at 20 °C, unreacted [γ-^{32}P]ATP was removed by gel filtration using the microcentrifuge desalting method. The isolated E~P^{32}P was then methylated with 5 mL of diazomethane solution (diazomethane/methanol, 9:1). After 30 min at 20 °C, another 5 mL of diazomethane was added and the solution was incubated for an additional 30 min. The resulting precipitate was recovered by brief centrifugation at low speed. The precipitate was washed 3 times with 10 mL of ethanol, and the pellet was removed as above. The pellet was then treated as described below.

S-Carboxymethylation and Digestion of the Methylated [^{32}P]Pyrophosphoryl-Enzyme. The methylated E~P^{32}P was dissolved in 6 M guanidine hydrochloride and was S-carboxymethylated by the method described by Beyreuther et al. (1975), except that iodoacetamide was replaced by iodoacetic acid. The salts were removed by dialysis against 0.1 M NH_4HCO_3 (3 × 4 L) for 16 h. The dialyzed protein was then digested at 37 °C for 6 h with trypsin (2% w/w). This was followed by digestion with α-chymotrypsin under the same conditions used for trypsin, except that digestion was for 16 h. The digest was lyophilized and redissolved in Tris–acetate (pH 8.5) and digested with leucine aminopeptidase (2% w/w)

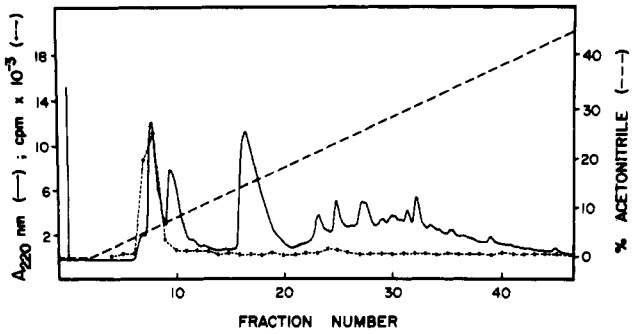

FIGURE 2: Reverse-phase HPLC of enzymic digest of diazomethylated [^{32}P]pyrophosphoryl pyruvate,phosphate dikinase. The digest was separated on a Synchropak reverse-phase C_{18} column (RP-P, 25 cm × 4.6 mm) by using a Du Pont Model 850 liquid chromatograph. The column was eluted isocratically for 2.5 min with 0.05% TFA at 0.9 mL/min. A linear gradient of 0–70% CH_3CN over 60 min was then applied by using 0.05% TFA and CH_3CN containing 0.05% TFA. The solid line (—) indicates absorption at 220 nm. Aliquots of each fraction were analyzed for radioactivity (-●-). The dashed line (--) represents the CH_3CN gradient.

for 24 h at 37 °C. Pronase (2% w/w) was then added and the mixture incubated for 24 h at 37 °C. The digest was lyophilized and dissolved in 0.05% TFA. Aliquots were fractionated on a reverse-phase C_{18} column by HPLC as described in the legend of Figure 2. The fractions were analyzed for radioactivity, and as seen in Figure 2, only one radioactive peak was observed. However, the profile of absorption at 220 nm indicated that the digestion was incomplete. The amino acid analysis of the lyophilized radioactive fraction showed the presence of a number of amino acids, resulting from incomplete digestion (results not shown).

Another aliquot of the radioactive fraction was freeze-dried and digested in 5% formic acid (pH 2) with pepsin (1%) for 24 h at 37 °C. After lyophilization, the digest was treated with PITC to enable the detection and purification of the ^{32}P-labeled residue. The treatment with PITC was as described for the amino acid analysis (see Methods). The PITC-treated and dried material was dissolved in 0.05% TFA and fractionated on a reverse-phase C_{18} ODS column by HPLC as described in the legend to Figure 3. After locating the peak with the highest amount of radioactivity (196 cpm in Figure 3), additional aliquots were fractionated as above and the desired fraction was recovered by manual collection, while monitoring the absorption at 254 nm, to eliminate the second peak (62 cpm). These fractions were combined and subjected to amino acid analysis to identify the pyrophosphorylated residue as described below. Other PTC-amino acids and byproducts eluted later in this system, and these fractions did not contain any radioactivity. The peaks with radioactivity of 32–43 cpm were within the range of background counts observed in samples with no 254-nm absorption.

Identification of Histidine as the Pyrophosphorylated Residue. To circumvent the nonavailability of an authentic marker for the methylated pyrophosphohistidine residue, the strategy employed was to remove the methyl (dimethyl or trimethyl) pyrophosphoryl moiety from the isolated radioactive residue by acid hydrolysis, followed by identification of the free amino acid residue. The PTC group linked to the primary amino group of histidine is also removed by this hydrolysis. Therefore, the isolated radioactive compound was acid-hydrolyzed and recoupled with PITC as described under Methods. The PTC-amino acids were then separated on a reverse-phase C_{18} ODS column by HPLC (Figure 4a) and identified by comparison with the retention times of the standard PTC-amino acids (Figure 4b). As seen from Figure

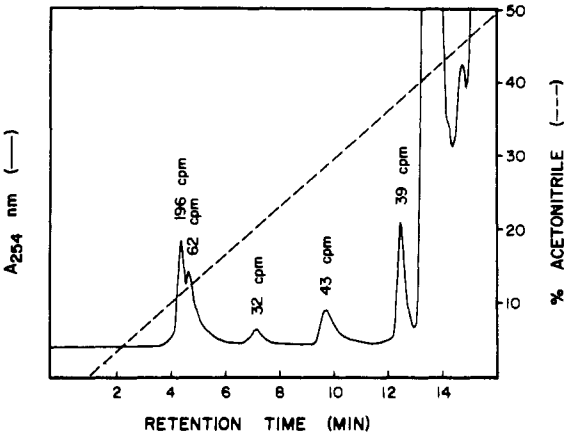

FIGURE 3: HPLC of radioactive fraction (from Figure 2) digested with pepsin and treated with PITC. The digest was treated with PITC, dried, dissolved in 200 μL of 0.05% TFA, and fractionated on a Beckman reverse-phase C_{18} ODS Ultrasphere column (25 cm × 4.6 mm, 5 μm). The column was eluted with 0.05% TFA for 2.5 min at 0.9 mL/min, followed by a linear gradient of 0–100% CH_3CN for 45 min. PTC-amino acids were monitored at 254 nm (—), and radioactive content of each peak was determined by counting an aliquot in scintillant. The values are displayed next to the peaks. The radioactive peak (peak 1, 196 cpm) was recovered by manual collection of the fractions from subsequent injections of the pepsin digest.

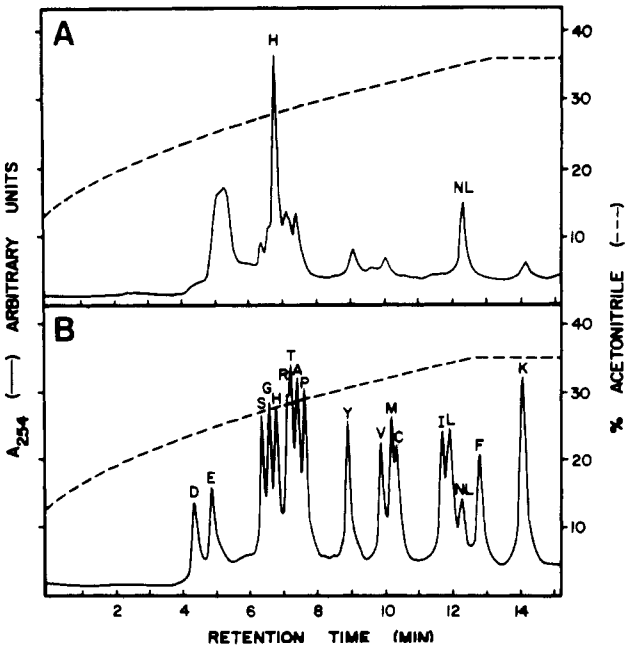

FIGURE 4: Identification of the pyrophosphorylated residue by reverse-phase HPLC. The lyophilized radioactive material (from Figure 3) was dissolved and hydrolyzed in 50 μL of 6 N HCl and derivatized with PITC as described under Methods. The dried samples along with 100 pmol of PTC-norleucine (NL) were dissolved in 20 μL of buffer A (see Methods) and fractionated on a Beckman C_{18} ODS Ultrasphere column (25 cm × 4.6 mm, 5 μm) by using a Waters HPLC system (panel A). Standard PTC-amino acids (50 pmol, Cys = 25 pmol) were derivatized as described under Methods and separated (panel B).

4a, the major amino acid was PTC-histidine with a relative retention time of 0.55 with reference to the internal standard, norleucine. The amount of PTC-histidine as quantitated from the peak area was 70% of the amount estimated from the radioactivity. The amounts of the other amino acids were well below the amount estimated from the radioactivity. The unidentified peak appearing just after PTC-Glu (relative retention time = 0.43) did not correspond to any of the amino acids. This peak probably represents some byproduct of the PITC

reaction with acid-hydrolyzed samples. We have occasionally observed this peak from acid hydrolysates of peptides, and almost certainly it is not a PTC-amino acid.

Discussion

Two lines of evidence support the earlier suggestions that the pyrophosphoryl moiety of E~PP is linked to a histidine residue: (A) isolation of a histidine residue from the hydrolysates of [^{32}P]pyrophosphoryl-enzyme, which had been stabilized with diazomethane, and (B) the hydrolytic pattern with hydroxylamine and pyridine.

Diazomethylated [^{32}P]Pyrophosphorylhistidine. All previous attempts to isolate the pyrophosphorylated amino acid residue from pyruvate,phosphate dikinase have failed because of the lability of the pyrophosphorylated derivative that is readily hydrolyzed to the phospho-enzyme (E~P). Only by stabilizing the pyrophosphoryl-enzyme through methylation has it been possible to obtain the amino acid residue containing the ^{32}P from [γ-^{32}P]ATP. It would have been more elegant if we could have demonstrated that the pyrophosphoryl moiety was on a histidyl group by comparison with authentic methylated pyrophosphorylhistidine. However, this was not possible since this compound is not available or is readily synthesized. Nevertheless, the identification of the hydrolyzed product as histidine (Figure 4) clearly shows that ^{32}P was associated with histidine. This is in accord with the observation that [β-^{32}P]ATP gives rise to [^{32}P]P-enolpyruvate (Milner et al., 1978), and [^{32}P]P-enolpyruvate yields the E~P form of the enzyme, identified as the $N^{3'}$-histidine phosphate (Yoshida & Wood, 1978). The precise structure of the diazomethylated histidyl pyrophosphate residue is not known although it is presumed to be the trimethyl derivative of the histidyl pyrophosphate. However, the mono- and dimethyl derivatives produced as a result of incomplete methylation are also possible.

An interesting feature of this pivotal histidyl residue is that threonine and serine occur adjacent to it (Goss et al., 1980). In addition, the reactivity of the pivotal histidine residue is affected by modification of a lysine residue with pyridoxal 5′-phosphate (Phillips et al., 1983). This reactive lysine is thought to be close to the histidine. Although the four amino acids including histidine are potentially phosphorylatable, the investigation by Spronk et al. (1976) and the present findings firmly support the view that histidine is the real acceptor of the phosphate and pyrophosphate moieties, and there is no indication of phosphorylated or pyrophosphorylated seryl, threonyl, or lysyl residues.

In the plant enzyme, a histidine residue and a threonine residue have been shown to be phosphorylated (Burnell & Hatch, 1984; Burnell, 1984). However, histidine-P results from catalytic phosphorylation while threonine-P is the result of inactivation-dependent regulatory phosphorylation. The proximity of the catalytically important histidine to the threonine residue important in the regulation is not known.

pH Stability of the Pyrophosphoryl Linkage. The profile in Figure 1 shows that the E~PP intermediate is labile at both pH extremes, with a narrow range of stability. The only hydrolysis curve that resembles the curve observed with E~P^{32}P is the hydrolytic curve for acyl phosphates. Acyl phosphates are labile at either pH extreme but reasonably stable at neutral pH, giving a U-shaped curve (Koshland, 1952; Black & Wright, 1955; Walsh & Spector, 1969). It is known that the histidylphosphoramidate is stable to base but acid labile, giving rise to a curve that starts with a high K_1 at low pH and falls to a low K_1 at high pH (Boyer et al., 1962; Spronk et al., 1976). We interpret the lability of the ^{32}P in the E-His^{3}N–P–O–^{32}P form at alkaline pH to be due to the hydrolysis of the pyrophosphophoryl linkage (P–O–P) and the lability at acid pH to the hydrolysis of the amidate linkage (N–P). It has been shown with succinyl phosphate that at alkaline pH (10.2) the hydrolysis of the anhydride bond proceeds largely by P–O bond rupture, but at pH 6.9, C–O cleavage of the anhydride predominates (Walsh et al., 1970). A similar situation might occur with the N–P and P–O–P bonds of the histidyl pyrophosphate linkage. Indeed, in acid conditions the [^{32}P]pyrophosphoryl-enzyme was hydrolyzed to both [^{32}P]-pyrophosphate and [^{32}P]orthophosphate, and under milder conditions (0.02 N HCl for 6 h at 21 °C), a higher percentage of radioactivity was observed as pyrophosphate (Milner & Wood, 1972).

None of the other phospho-amino acid derivatives have hydrolysis curves resembling that of E~PP. Phosphate esters of serine and threonine are acid stable but alkali labile (Plimmer & Bayliss, 1906). The stability of *O*-phosphotyrosine to base is similar to that of phosphoramidate linkages; however, Tyr-P is stable to acidic conditions that destroy *N*-phosphates (Plimmer, 1941). Thiophosphates (of cysteine) are quite stable at very high or very low pH but labile in slightly acidic conditions (Pigiet & Conley, 1978). The pH profile in this case would show an inverted U shape.

Effect of Hydroxylamine and Pyridine on Pyrophosphoryl-Enzyme. The data obtained from this study clearly show that E~P^{32}P does not have the characteristics of an acyl phosphate. The model compound for acyl phosphoproteins, acetyl phosphate, has a k_2 of 41.8 mol^{-1} min^{-1} with hydroxylamine, but there is very little hydrolysis by pyridine, the k_2 being 0.0087 for the divalent phosphate species and 0.046 on the monovalent species at 39 °C (Disabato & Jencks, 1961). The data from Table I resemble the hydrolytic pattern of the phosphoramidate compound. By analogy with the phosphoramidate linkage of E~P, it is considered that the release of the ^{32}P from [γ-^{32}P]ATP-labeled enzyme by pyridine establishes the bond as an amidate and not an acyl phosphate. This evidence, combined with the proof that the $^{32}P_i$ is linked to a histidyl group, establishes the bonding as E-HisN–P–O^{32}P.

Prior to these lines of evidence, the only indication that the pyrophosphoryl moiety was linked to a histidine residue has come from studies on the acid lability of the E~PP (Milner et al., 1978). They demonstrated that when the isolated E~PP was subjected to mildly acidic conditions, pyrophosphate was released, indicating that it was covalently linked to a histidyl residue. Additional support for the involvement of a histidine in the pyrophosphoryl linkage has come from the observation that ATP (which forms E~PP) retarded the rate of inactivation by diethyl pyrocarbonate, a histidine-specific reagent (Yoshida & Wood, 1978). Previous attempts to isolate a [^{32}P]pyrophosphohistidine by the classical methods of alkaline hydrolysis have failed (Spronk et al., 1976), the reason being obvious from the observation that the pyrophospho-enzyme is very labile under alkaline conditions (Figure 1).

Other Possible Pyrophosphoryl-Enzymes. Recent stereochemical studies on P-enolpyruvate synthetase and pyruvate,phosphate dikinase (Cook & Knowles, 1985) have yielded evidence that both enzymes involve phospho- and pyrophospho-enzyme intermediates. The kinetic studies of Berman and Cohn (1970) indicated that a free pyrophospho-enzyme in the P-enolpyruvate synthetase was improbable although the initial proposal included a pyrophospho-enzyme intermediate in the reaction sequence (Cooper & Kornberg, 1967a–c).

The mechanism proposed by Andrews and Hatch (1969) for pyruvate,phosphate dikinase isolated from tropical grass,

sugar cane, maize, and sorghum is similar to that proposed by Berman and Cohn (1970) for P-enolpyruvate synthetase from *Escherichia coli*, except that in the latter case water replaces orthophosphate in reaction 2a. These results, however, do not necessarily exclude the transient formation of a pyrophosphoryl-enzyme intermediate. If the E~PP intermediate does exist, it might be possible to trap and demonstrate its existence by the methods described here. In light of the findings of Cook and Knowles (1985), similar attempts could be made to demonstrate the pyrophospho-enzyme intermediate from P-enolpyruvate synthetase. We have no explanation for the failure of Dunaway-Mariano and co-workers (Ciskanik & Dunaway-Mariano, 1983; Wang et al., 1984) to observe the pyrophosphoryl form of pyruvate,phosphate dikinase from *B. symbiosus*.

ACKNOWLEDGMENTS

We thank Maxwell Adams for technical assistance.

Registry No. Pyrophosphohistidine, 100764-00-9; histidine, 71-00-1; pyruvate,phosphate dikinase, 9027-40-1.

REFERENCES

Andrews, T. J., & Hatch, M. D. (1969) *Biochem. J. 114*, 117.
Berman, K. M., & Cohn, M. (1970) *J. Biol. Chem. 245*, 5319.
Beyreuther, K., Adler, K., Fanning, E., Murray, C., Klemm, A., & Geisler, N. (1975) *Eur. J. Biochem. 59*, 491.
Black, S., & Wright, N. G. (1955) *J. Biol. Chem. 213*, 27.
Boyer, P. D., Deluca, M., Ebner, K. E., Hultquist, D. E., & Peter, J. B. (1962) *J. Biol. Chem. 237*, 3306.
Burnell, J. N. (1984) *Biochem. Biophys. Res. Commun. 120*, 559.
Burnell, J. N., & Hatch, M. D. (1984) *Arch. Biochem. Biophys. 231*, 175.
Ciskanik, L., & Dunaway-Mariano, D. (1983) *Biochemistry 22 (15)*, 7A (Abstr. 12).
Cook, A. G., & Knowles, J. R. (1985) *Biochemistry 24*, 51.
Cooper, R. A., & Kornberg, H. L. (1967a) *Biochim. Biophys. Acta 141*, 211.
Cooper, R. A., & Kornberg, H. L. (1967b) *Biochem. J. 105*, 49c.
Cooper, R. A., & Kornberg, H. L. (1967c) *Proc. R. Soc. London, B 168*, 263.
Disabato, G., & Jencks, W. P. (1961) *J. Am. Chem. Soc. 83*, 4393.
Evans, H. J., & Wood, H. G. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1448.
Evans, H. J., & Wood, H. G. (1971) *Biochemistry 10*, 721.
Goss, N. H., Evans, C. T., & Wood, H. G. (1980) *Biochemistry 19*, 5805.
Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65.
Helmerhorst, E., & Stokes, G. B., (1980) *Anal. Biochem. 104*, 130.
Jencks, W. P., & Gilchrist, M. (1965) *J. Am. Chem. Soc. 87*, 3199.
Koshland, D. E., Jr. (1952) *J. Am. Chem. Soc. 74*, 2286.
Milner, Y., & Wood, H. G. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2463.
Milner, Y., & Wood, H. G. (1976) *J. Biol. Chem. 251*, 7920.
Milner, Y., Michaels, G., & Wood, H. G. (1978) *J. Biol. Chem. 253*, 878.
Phillips, N. F. B., Goss, N. H., & Wood, H. G. (1983) *Biochemistry 22*, 2518.
Pigiet, V., & Conley, R. R. (1978) *J. Biol. Chem. 253*, 1910.
Plimmer, R. H. A. (1941) *Biochem. J. 35*, 461.
Plimmer, R. H. A., & Bayliss, W. M. (1906) *J. Physiol. (London) 33*, 439.
Powers, S. G., & Meister, A., (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3020.
Spronk, A. M., Yoshida, H., & Wood, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4415.
Symons, R. H. (1977) *Nucleic Acids Res. 4*, 4347.
Tarr, G. E. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., Ed.) pp 223–232, Humana, Clifton, NJ.
Vogel, I. A. (1967) in *Text Book of Practical Organic Chemistry*, 3rd ed., p 967, Longmans, Green & Co., London.
Walsh, C. T., & Spector, L. B. (1969) *J. Biol. Chem. 244*, 4366.
Walsh, C. T., Hildebrand, J. G., & Spector, L. B. (1970) *J. Biol. Chem. 245*, 5699.
Wang, H.-C., Ciskanik, L., & Dunaway-Mariano, D., (1984) *Fed. Proc, Fed. Am. Soc. Exp. Biol. 43*, 2137 (Abstr.).
Yoshida, H., & Wood, H. G. (1978) *J. Biol. Chem. 253*, 7650.