Isolation of 3-phosphohistidine from phosphorylated pyruvate, phosphate dikinase

(prophosphoenzyme/phosphoenzyme/transphosphorylation/phosphoeno[14C]pyruvate/Bacteroides symbiosus)

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Contributed by Harland G. Wood, September 17, 1978

ABSTRACT Pyruvate, phosphate dikinase (EC 2.7.9.1) catalyzes formation of phosphoeno[14C]pyruvate, AMP, and inorganic phosphate from pyruvate, ATP, and orthophosphate. A pyrophosphoryl and phosphohydrolysis form of the enzyme is involved in this transfer. The 32P-phosphoryl form of pyruvate, phosphate dikinase was prepared with enzyme isolated from Bacteroides symbiosus. The 32P-phosphoryl form was found to have properties corresponding to a phosphoramidine linkage and this was confirmed by isolation of 32P-phosphohistidine from alkaline hydrolysates of the enzyme. The histidyl residue is considered to be the pyrophosphoryl- and phosphoryl-carrier between the three substrate sites of this enzyme.

Pyruvate, orthophosphate dikinase (EC 2.7.9.1) catalyzes the following reaction:

\[ \text{ATP} + \text{pyruvate} + \text{P}_i \rightarrow \text{AMP} + \text{P-enol}^{} \text{pyruvate} + \text{PP}_i \]  

[1]

The overall reaction involves three partial reactions:

\[ \text{ATP} + \text{enzyme} \rightarrow \text{pyrophosphoryl enzyme} + \text{AMP} \]  

[1a]  

\[ \text{Pyrophosphoryl enzyme} + \text{P}_i \rightarrow \text{phosphoryl enzyme} + \text{PP}_i \]  

[1b]  

\[ \text{Phosphoryl enzyme} + \text{pyruvate} \rightarrow \text{enzyme} + \text{P-enolpyruvate} \]  

[1c]  

Previous reports (1, 2) have demonstrated the presence of phosphoryl and pyrophosphoryl forms of this enzyme, using enzyme preparations from Propionibacterium shermanii. These data and detailed kinetic analysis of the three partial reactions and exchange reactions (3, 4) have demonstrated that the pyruvate, phosphate dikinase reaction occurs by a three-site nonclassical Tri (Uni Uni) Ping Pong mechanism. A pyrophosphoryl- and phosphoryl-histidyl residue of the enzyme has been proposed to serve as a carrier between the three sites (4).

A number of enzymes that have reaction mechanisms involving phosphate moieties covalently bound to enzyme are now known (5). The phosphate has been found linked to proteins through different types of linkage, including a phosphoester with the hydroxyl group of serine (6), a phosphoramidate to N-1 or N-3 of histidine (7-10), and an acid anhydride to a y-carboxyl group of glutamic acid (11).

The phosphoramidate and acid anhydride type of bond may be considered high in chemical energy (12), and thus either of these might meet the requirement of phosphoryl pyruvate, phosphate dikinase. However, at the outset we could not exclude the possibility of ester linkage to serine because the phosphate of this type of bond has been known to be transferable to ADP with concomitant formation of ATP (13). Thus, in an appropriate micro-environment even a seryl-phosphate bond may be considered a “high energy” type.

In the present work using pyruvate, phosphate dikinase from Bacteroides symbiosus, the type of covalent bond of phosphoryl enzyme has been investigated and it has been shown to be a N-3-phosphohistidyl residue of the enzyme.

MATERIALS AND METHODS

Chemicals. [32P]Orthophosphate was purchased from New England Nuclear; P-enol[2-14C]pyruvate was a gift from Dr. W. E. O’Brien; diphenylchloro phosphate was from Aldrich; pyruvate kinase and lactate dehydrogenase were from Boehringer; and all other chemicals were of analytical grade from various firms.

Synthesis of [32P]P-enolpyruvate. [32P]P-enolpyruvate was synthesized from [32P]orthophosphate and β-chloralactate by scaling up the procedure of Lauppe et al. (14). The product was purified by chromatography on a Dowex 1-X8 column (Cl−, 2 × 25 cm) (1). The radioactive fractions were pooled, lyophilized, and stored frozen. The concentration of P-enolpyruvate was determined enzymatically by a pyruvate kinase–lactate dehydrogenase system and specific radioactivity was calculated in cpm/μmol by dividing the cpm in material that migrated on paper chromatography with authentic P-enol[2-14C]pyruvate by the μmol of P-enolpyruvate in the aliquot. The product was 92-94% pure.

Synthesis of Phosphohistidine. Phosphohistidine was synthesized by reacting potassium phosphoramidate and histidine (15). Phosphoramidate was prepared from diphenylchlorophosphate (16). The phosphohistidine was purified by chromatography on a 2.4 × 30 cm column of Dowex 1-X8 using a 2-liter linear gradient of 0–1.0 M KHCO3, pH 8.25. The fractions were assayed for histidine (17) and phosphate (18), following cleavage of the phosphoramidate by acidification of an aliquot to pH 1.0. The fractions were pooled, cooled in ice, and brought to pH 3.0 with perchloric acid. After CO2 evolution ceased, the pH was rapidly adjusted to 9.0 with potassium hydroxide, and the sample was filtered to remove the precipitated potassium perchlorate and lyophilized. Upon rechromatography the product (with some potassium perchlorate) gave a single peak, which was 3-phosphohistidine, as confirmed by chromatography of the product with a tracer amount of a mixture of L- and 3-phospho[14C]histidine that had been prepared according to Hays et al. (10).

Purification and Assay of Pyruvate, Phosphate Dikinase. The enzyme was purified from cells of Bacteroides symbiosus by the method of Milner et al. (19) and was stored at 4° as a precipitate in 80% saturated ammonium sulfate. It was pure by the criteria of disc gel electrophoresis, sedimentation ultracentrifugation, and equilibrium ultracentrifugation and had

Abbreviation: P-enolpyruvate, phosphoenolpyruvate.

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a specific activity of 18 μmol/min per mg, which with time decreased to 12 μmol/min per mg (19). Before use, the enzyme was dialyzed overnight against 20 mM imidazole-HCl buffer, pH 6.8, containing 75 mM KCl, 2.5 mM EDTA, and 3 mM 2-mercaptoethanol. The enzyme activity was assayed by measuring the amount of pyruvate formed from P-enolpyruvate (19). Protein concentration was determined by measuring the absorbance at 280 nm using an absorbance of 1.0 for a 1.3 mg/ml protein solution (19).

Formation of [32P]Phosphoryl Pyruvate, Phosphate Dikinase. [32P]Phosphoryl pyruvate, phosphate dikinase was synthesized via partial reaction 1c using [32P]P-enolpyruvate. Excess [32P]P-enolpyruvate was removed by Sephadex G-50 gel filtration. This procedure had been used previously for synthesis of the phosphoryl form of pyruvate, phosphate dikinase obtained from Propionibacterium shermanii (1, 2).

The molecular weight of the pyruvate, phosphate dikinase is about 160,000 and is dimeric (19). Acrylamide gel electrophoresis in sodium dodecyl sulfate gives a single band of molecular weight 75,000 (19). Usually only 1 mol of [32P]P-enolpyruvate was incorporated per mol of dimeric enzyme, although 2 mol are incorporated if a high concentration of [32P]P-enolpyruvate is employed. About a 2-fold excess of [32P]P-enolpyruvate was used, assuming 2 mol/mol of enzyme, and lactate dehydrogenase and DPNH were included to remove the pyruvate and shift the equilibria toward phosphoryl enzyme. A typical preparation contained 50 nmol of [32P]P-enolpyruvate (106 cpm/nmol), 1.6 mg of enzyme (10 nmol), 5 μg of lactate dehydrogenase, 120 nmol of DPNH in 0.3 ml of 0.05 M imidazole buffer, pH 6.8, containing 4 mM NaN3Cl and 0.5 mM MgCl2. Incubation was for 20 min at 25°. The solution was passed through a 1 × 12 cm column of Sephadex G-50 (fine) equilibrated with 20 mM imidazole buffer (pH 6.8) containing 75 mM KCl, 2.5 mM EDTA, and 3 mM 2-mercaptoethanol. The phosphoryl protein is quite stable with respect to the phosphate linkage, having a half-life of 6–9 days at 4°.

Paper Chromatography of 3-Phosphohistidine and of P-enolpyruvate. The solvent for 3-phosphohistidine was n-propanol/ethanol/H2O/ammonia (30:30:39:1) (20) and the paper was sprayed with a 0.5% ninhydrin solution after the run. For P-enolpyruvate the solvent was methanol/ammonia/H2O (60:10:30) (21).

Radioactivity was measured by liquid scintillation with a Unilux I counter (Nuclear Chicago).

RESULTS

Evidence that the 32P was covalently linked to the pyruvate-phosphate dikinase was obtained by gel isoelectric focusing (Fig. 1). Better than 95% of the recovered radioactivity was associated with the major band of protein which had migrated to pH 5.2. A small amount of radioactive material migrated nearly to the bottom of the gel. The appearance of this band was due to some hydrolysis of the phosphoryl enzyme at the acid pH, thus yielding orthophosphate.

pH Stability of the Phosphoryl Linkage. In order to characterize the covalently bound phosphate, the pH stability profile was determined. These results indicated conditions to be used for hydrolysis of the phosphoryl enzyme and isolation of phospho-amino acid. In addition, the stability profiles of various phospho-amino acid bonds are known from study of model compounds and comparison of these profiles with that of the phosphoryl enzyme provided an indication of the type of bond occurring in the phosphoprotein. Phosphate esters of hydroxy amino acids are stable at pH values between 1 and 14 (23); phosphoramidate linkages to histidine residues are labile under acidic conditions but stable in base (15, 16); acyl phosphates are labile at both high and low pH values and reasonably stable at neutral pH (24–26). An enzyme containing a phospho-thioester would probably behave like the model compound 3-phospho-4-thiobutane in solution, which is quite stable under moderately acidic conditions and at pH values greater than 7, with a peak of lability occurring at pH values between 3 and 5 (27). It is noted that phosphate linked to tyrosine or cysteine has not as yet been reported to occur in phosphoprotein. Neither has phosphosine or phosphoarginine been found in nature; it is expected that the stability characteristics of the latter two compounds would be like phosphoramidate.

The first-order rate constants of hydrolysis, k1, of the phosphoenzyme at different pH values are shown in Fig. 2. It is clear that the linkage is very labile under acidic conditions and very stable under basic conditions. In fact, in 0.01 M carbonate buffer, pH 10, the half-life exceeded 60 days. No data were obtained for the region of pH between 2 and 5.5 because the protein precipitated under these conditions in both citrate-phosphate and acetate buffers, making it impossible to study stability in that pH range using the present technique.

Effect of Hydroxylamine and Pyridine on Phosphoryl Pyruvate, Phosphate Dikinase. The pH lability of phosphoryl pyruvate, phosphate dikinase is characteristic of a phosphoramidate-type bond (15). The possibility of an acyl phosphate could not be completely excluded, however, because of the slight increase in k1 at pH 13.5. It is known that at pH values greater than 13.5 base-catalyzed hydrolysis of the acyl phosphate of acetate kinase occurs at a significant rate (28). In addition, phosphate migration from an acyl group to a histidyl group was observed at higher pH values by Nazarova et al. (29) with yeast pyrophosphatase. Thus, in order to obtain additional support for the occurrence of a phosphoramidate-type linkage of the phosphoryl pyruvate, phosphate dikinase, the enhancement of hydrolysis by hydroxylamine and pyridine was studied (Fig. 3). The second-order decay constants were calculated using the equation k2 = kobs − k1/[catalyst], kobs is the apparent first-order decay constant of the phosphoryl enzyme with catalyst (hydroxylamine or pyridine) and k1 without catalyst (calculated as in Fig. 2). Since only the free base is catalytically
active (30), the correct concentration of this active species was calculated by use of the Henderson—Hasselbach equation, using the final pH and the known added concentration of catalyst.

The apparent second-order rate constants $k_2$ for phosphoryl pyruvate,phosphate dikinase hydrolysis were 0.50 and 0.25 mol$^{-1}$ min$^{-1}$ for hydroxylamine and pyridine, respectively. These data are in reasonably good agreement with the kinetic constants of 0.8 and 0.7 mol$^{-1}$ min$^{-1}$, respectively, reported for phosphoramidate hydrolysis at 30°C (30). Acetyl phosphate may be considered a model compound for comparison of acyl phosphoproteins. It has a $k_2$ of 42 mol$^{-1}$ min$^{-1}$ with hydroxylamine but there is very little stimulation of the hydrolysis by pyridine, the $k_2$ being 0.009 for the attack of pyridine on the divalent phosphate species and 0.046 on the monovalent species (31). Thus, there are about 4 orders of magnitude between the $k_2$ values of hydroxylamine- and pyridine-catalyzed hydrolyses of acyl phosphate. The $k_2$ values from our data differ only by a factor of 2, and are near those reported for the model compound phosphoramidate. Thus, the data are in accord with the phosphate's being bound to pyruvate,phosphate dikinase via a phosphoramidate bond. Hays et al. (10) determined second-order rate constants of 0.55 and 0.43 for hydroxylamine and pyridine, respectively, for dephosphorylation of phosphorylated III$^{lac}$ protein, which was, with additional studies, also shown to have its phosphate bound as 3-phosphohistidine.

Isolation of $[^{32}P]$Phosphohistidine from $[^{32}P]$Phosphoryl Pyruvate,Phosphate Dikinase. The above results established that the phosphoryl pyruvate,phosphate dikinase has the properties of a phosphoramidate, which might occur in a linkage to a nitrogen of lysine, arginine, or histidine residue of the protein. We focused our attention on phosphohistidine because diethylypyrocarbonate, which is known as a specific modifying reagent of histidine (32), inactivated the enzyme (H. Yoshida and H. G. Wood, unpublished data). Yoshida and Wood found (unpublished data) 18 histidyl residues per mol of enzyme reacted with the diethylypyrocarbonate when the unphosphorylated enzyme was used and only 16 with the phosphoryl enzyme. These results indicate two histidyl groups are phosphorylated and blocked from reaction with the reagent.

$[^{32}P]$Phosphoryl pyruvate,phosphate dikinase was subjected to hydrolysis with 3 M NaOH. The phosphoramidate linkage is reasonably stable under these conditions. Fig. 4 shows the
result of co-chromatography of the hydrolysate to which carrier 3-phosphohistidine and tracer amounts of 1-phospho- and 3-phospho-[carboxyl-14C]histidine were added. There were two peaks of 32P radioactivity, the first corresponding to the position of inorganic phosphate (tube 33) and the second to the second half of the double peak of 1- and 3-phospho-[carboxyl-14C]histidine. This order of their elution is well established (10). No 32P was associated with 1-phospho-[carboxyl-14C]histidine. If the phosphate was on N-1 originally, it would be expected that a part but not all would have migrated to N-3 of another histidine residue in the hydrolysate so that a mixture of 1- and 3-[32P]phosphohistidine would be found after chromatography of the hydrolysate (10). Further evidence that the labeled compound was a phosphohistidine was obtained by paper chromatography (20) of the Dowex column eluate (Fig. 5). The radioactive peak corresponded to the peak of authentic 3-phosphohistidine. Thus, it is clear that a phosphoramidate linkage is formed between phosphate and a histidine residue of the active site.

DISCUSSION

The reaction mechanism involving phosphoryl and pyrophosphoryl forms of pyruvate-phosphate dikinase from Bacteroides symbiosus, as determined by careful kinetic studies (Y. Milner and H. G. Wood, unpublished data), appears to be similar to that already proposed for the Propionibacterium shermanii enzyme (4). However, kinetic data per se are not sufficient proof for the existence of the phosphoryl enzyme form. The isoelectric focusing of phosphoryl pyruvate-phosphate dikinase and the isolation of 3-phosphohistidine unequivocally identify the phosphoryl form of the enzyme. Three lines of evidence support this conclusion: (i) pH stability profile, (ii) susceptibility to pyridine and hydroxylamine hydrolysis, and (iii) isolation of only 3-[32P]phosphohistidine from hydrolysates of [32P]phosphoryl pyruvate-phosphate dikinase. A number of enzymes have been reported to involve 3-phosphohistidine in their reaction mechanism, namely, succinate thiokinase (33), lactose-specific (lac III) phosphocarrier protein of the Staphylococcus aureus phosphotransferase system (10), microsomal glucose-6-phosphatase (34), and wheat germ and human prostate acid phosphatase (35). There is no evidence that the phosphoryl form of pyruvate-phosphate dikinase is less stable than the free enzyme; no decrease in specific activity of the enzyme was found during storage in the phosphoryl form in imidazole-HCl buffer at 4°C. Frequently no loss of specific activity was evident for 30 days.

1-Phosphohistidine has been found to occur in phosphate transfer reactions catalyzed by nucleotide diphosphate kinase (36) and Hpr, the phosphoenolpyruvate-dependent phosphocarrier protein of Escherichia coli (9) and Staphylococcus aureus (37).

Evidence presented by Milner and Wood (4) shows that the reaction mechanism of pyruvate-phosphate dikinase is via a nonclassical three-site Tri (Uni Uni) Ping Pong mechanism with separate ATP-AMP, P1-PP, and pyruvate-P-enolpyruvate sites. Accordingly, the pyrophosphoryl group of the ATP is transferred at the ATP-AMP site to a histidyl group of the enzyme. The pyrophosphoryl histidyl group is then transferred to the P1-PP site, where one phosphate of the pyrophosphohistidyl group combines with P1, forming PPi and the phosphohistidyl enzyme. The phosphohistidyl group is then transferred to the pyruvate-P-enolpyruvate site, where the phosphate of the phosphohistidyl group is transferred to pyruvate, forming P-enolpyruvate and the unphosphorylated enzyme. These transfers to the sites may occur via conformational changes involving movement of a flexible peptide chain containing the pyrophospho- and phosphohistidyl groups.

The pyrophosphoryl form of pyruvate-phosphate dikinase from Bacteroides symbiosus is unstable and all attempts to isolate 5-[32P]pyrophosphohistidine from hydrolysates of the [32P]pyrophosphoryl enzyme have failed. Michaels et al. (38) reported that pyrophosphoryl enzyme synthesized by partial reaction Ia undergoes a slow hydrolysis to give phosphoryl enzyme plus phosphate. Phosphate produced in this reaction is free to react with pyrophosphoryl enzyme to give phosphoryl enzyme plus pyrophosphate. Thus, the breakdown would be autocatalytic.

This work was supported by Grant BMS 74-22792 from the National Institutes of Health.