Pyruvate Phosphate Dikinase: Sequence of the Histidyl Peptide, the Pyrophosphoryl and Phosphoryl Carrier†

Neil H. Goss, Claudia T. Evans,‡ and Harland G. Wood*

ABSTRACT: Pyruvate phosphate dikinase contains a pivotal histidyl residue which functions to mediate the transfer of phosphoryl moieties during the reaction catalyzed by the enzyme. The tryptic peptide which contains this essential histidyl residue has been isolated by a two-step procedure originally developed by Wang and co-workers [Wang, T., Jurasek, L., & Bridger, W. A. (1972) *Biochemistry 11*, 2067]. This peptide has been sequenced by the manual dansyl-Edman procedure and is shown to be NH_2-Gly-Gly-Met-Thr-Ser-His-Ala-Ala-Val-Val-Ala-Arg-CO_2H. There is no readily interpretable homology between this peptide and other phosphorylated histidyl peptides previously isolated from other enzymes. By use of Chou & Fasman [Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222], it is predicted that the sequence contains an α helix from the methionine residue through to the carboxyl terminal arginine residue.

The reaction catalyzed by pyruvate phosphate dikinase (EC 2.7.9.1) involves the interconversion of pyruvate and phosphoenolpyruvate as shown in eq 1. Studies of the enzyme

$$\text{ATP} + \text{P}_\text{i} + \text{pyruvate} \rightleftharpoons \text{AMP} + \text{PP}_\text{i} + \text{phosphoenolpyruvate} \quad (1)$$

isolated from either *Propionibacterium shermanii* (Evans & Wood, 1968; Milner & Wood, 1972) or *Bacteroides symbiosus* (Milner et al., 1978) have shown that both a phosphoryl-enzyme intermediate (E-P)[1] and a pyrophosphoryl-enzyme intermediate (E-PP) are formed during the reaction as illustrated in eq 1a–c.

$$\text{E} + \text{ATP} \rightleftharpoons \text{E}\text{-}\overset{\beta}{\text{P}}\overset{\gamma}{\text{P}} + \text{AMP} \quad (1a)$$

$$\text{E}\text{-}\overset{\beta}{\text{P}}\overset{\gamma}{\text{P}} + \text{P}_\text{i} \rightleftharpoons \text{E}\text{-}\overset{\beta}{\text{P}} + \overset{\gamma}{\text{P}}\text{P}_\text{i} \quad (1b)$$

$$\text{E}\text{-}\overset{\beta}{\text{P}} + \text{pyruvate} \rightleftharpoons \text{E} + \overset{\beta}{\text{P}}\text{hosphoenolpyruvate} \quad (1c)$$

Characterization of the phosphoryl-enzyme intermediate indicated that the phosphate moiety was bound to the enzyme through an acid-labile phosphoramidate linkage and subsequently led to the isolation of *N*-3-[^{32}P]phosphohistidine from alkaline hydrolysates of [^{32}P]phosphoryl-enzyme (Spronk et al., (1976). The pyrophosphoryl-enzyme intermediate has also been isolated and Milner et al. (1978) demonstrated that when the pyrophoshoryl-enzyme was subjected to mildly acidic conditions, pyrophosphate was released, indicating that it was covalently linked to the histidyl residue of the enzyme. However, instability of the pyrophosphoryl-enzyme has prevented the isolation and identification of the pyrophosphoryl histidyl moiety.

The involvement of histidine as the pyrophosphoryl and phosphoryl acceptor has been confirmed by Yoshida & Wood (1978) using diethyl pyrocarbonate as a chemical modifier of pyruvate phosphate dikinase. The essential histidyl residue is considered to be centrally located in the active site of the enzyme and functions to mediate the transfer of the phosphoryl and pyrophosphoryl moieties between the three partial reaction subsites. It is interesting to note that the reaction catalyzed by the apparently closely related enzyme phosphoenolpyruvate synthetase from *Escherichia coli* also involves the intermediate participation of a 3-phosphohistidine (Suree & Bridger, 1977, 1978).

The objective of the present study was to isolate and sequence the phosphohistidyl peptide produced from tryptic digestion of pyruvate phosphate dikinase. Such information, when combined with other studies, can provide a more detailed understanding of the events occurring in the active site of the enzyme at the molecular level. In addition, the sequences around the phosphohistidine residues in the active sites of a variety of other phosphotransferase enzymes are now available (Wang et al., 1972; Rose et al., 1975; Beyreuther et al., 1977; Han & Rose, 1979), and, by comparison of these sequences with the sequence obtained from pyruvate phosphate dikinase, an evaluation of any evolutionary relationships between these enzymes should be possible.

Experimental Procedures

Materials

[^{32}P]Phosphoenolpyruvate was prepared according to the method of Lauppe et al. (1972) by using [^{32}P]orthophosphate obtained from New England Nuclear. Polyamide plates were obtained from Schleicher & Schuell, TPCK–trypsin was from Worthington Biochemical Corp., carboxypeptidase B was from Sigma Chemical Co., DEAE-cellulose (DE-52) was from Whatman, Inc., and phenyl isothiocyanate and trifluoroacetic acid were from Pierce Chemical Co. All other chemicals were reagent grade or better.

Methods

Preparation of Pyruvate Phosphate Dikinase from B. symbiosus. Pyruvate phosphate dikinase was prepared from *B. symbiosus* cells grown on glucose media by a method similar to that described by South & Reeves (1975).

Preparation of Cell-Free Extract. Cells (120 g) were suspended at 37 °C with vigorous stirring in 950 mL of 20 mM imidazole–HCl buffer, pH 6.8, containing 75 mM KCl, 2.5 mM EDTA, and 3.0 mM 2-mercaptoethanol. Lysis of the cells was achieved by the addition of lysozyme (1 mg/g of cells) to the vigorously stirred suspension and incubation for 90 min at 37 °C. Streptomycin sulfate (0.1 g/g of cells) was then added and the solution stirred at 37 °C for a further 30

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[1] Abbreviations used: E-P, phosphoryl-enzyme intermediate; E-PP, pyrophosphoryl-enzyme intermediate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; TPCK, tosylphenylalanine chloromethyl ketone; $NaDodSO_4$, sodium dodecyl sulfate; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-phosphate.

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min. The suspension was clarified by centrifuging at 20000*g* for 20 min and the supernatant removed and stored at 4 °C. The pellet was resuspended in 250 mL of the same buffer and centrifuged as described above. The supernatants of the two centrifugations were combined, and all subsequent operations were performed at 4 °C.

Ammonium Sulfate Fractionation. To the combined supernatants was added solid ammonium sulfate to a concentration of 50% saturation, and the precipitated protein was removed by centrifuging at 20000*g* for 20 min. Pyruvate phosphate dikinase activity was precipitated from the supernatant by the addition of solid ammonium sulfate to a final saturation of 62%, and the precipitate was collected by centrifugation as described above. The precipitate was redissolved in 20 mM imidazole–HCl, pH 6.4, 2.5 mM EDTA, 0.6 mM 2-mercaptoethanol, and 88 mM KCl and dialyzed overnight against the same buffer at 4 °C.

DEAE-cellulose Chromatography. The dialyzed solution was applied to a DEAE-cellulose column (2.5 × 35 cm) equilibrated with the above buffer and eluted with an 800-mL gradient of KCl from 88 to 500 mM. The enzyme eluted at ~200 mM KCl.

Hydroxylapatite Chromatography. The fractions containing the active enzyme from the DEAE-cellulose column were pooled and applied directly to an hydroxylapatite column (3.0 × 40 cm) equilbrated with 200 mM imidazole–HCl, pH 6.4, 20 mM NH_4Cl, and 0.7 mM 2-mercaptoethanol. The column was then eluted with a 500-mL linear gradient of potassium phosphate from 0 to 400 mM, in the above buffer. The enzymic activity is associated with the first protein peak to elute from the column at ~175 mM potassium phosphate. The fractions containing the enzymic activity were pooled and precipitated with 80% saturated ammonium sulfate.

Chromatography on Bio-Gel A-0.5m. As a final purification step, the enzyme obtained from hydroxylapatite chromatography was redissolved in 20 mM imidazole–HCl, pH 6.5, containing 0.1 mM EDTA, 0.7 mM 2-mercaptoethanol, and 100 mM KCl and applied to a Bio-Gel A-0.5m column (2.5 × 90 cm) equilibrated in the same buffer. The enzymic activity eluted as a symmetrical peak coincident with the major protein peak and well resolved from the minor, lower molecular weight contaminants. The fractions containing the enzyme were pooled, precipitated with 80% saturated ammonium sulfate, and stored at 4 °C as an ammonium sulfate suspension.

The enzyme preparation was homogeneous as judged by $NaDodSO_4$ gel electrophoresis (Figure 1a), exhibiting a single protein band of molecular weight of 94000 (Figure 1b). When chromatographed on calibrated Sephacryl S-200 and Bio-Gel A-0.5m columns, the enzyme exhibited a molecular weight of ~170000, indicative of the dimeric structure of the enzyme (Figure 1c). By use of the reacting enzyme sedimentation technique of Cohen et al. (1967), and $s_{20,w}$ value equal to 9.6 S was found for the enzyme, and no active monomer was detected. These values are in contrast to those presented by Milner et al. (1975) who reported a molecular weight for the monomer in $NaDodSO_4$ of 75000, with a dimer molecular weight of 150000–160000 and a $s_{20,w}$ value equal to 8 S. We are unable to explain the discrepancy between our values and those reported earlier although it is possible that since the earlier preparations were performed in large scale over a period of several weeks, some proteolysis may have occurred, lowering the molecular weight of the enzyme, but having no effect on the enzymatic activity.

Preparation of the Phosphorylated Enzyme. Pyruvate phosphate dikinase was phosphorylated by using [^{32}P]-

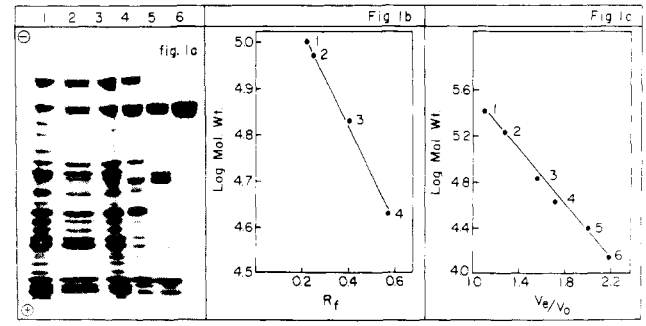

FIGURE 1: (a) Purification of pyruvate phosphate dikinase. Aliquots were taken at each stage of the enzyme preparation and subjected to electrophoresis on a 7.5% polyacrylamide slab gel in the presence of 0.1% $NaDodSO_4$. (1) cell lysate; (2) 50% ammonium sulfate supernatant; (3) 50–62% ammonium sulfate precipitate; (4) pool off of DEAE-cellulose chromatography; (5) pool off of hydroxylapatite chromatography; (6) pool off of Bio-Gel A-0.5m. (b) Molecular weight of the monomer of pyruvate phosphate dikinase. The standard proteins were electrophoresed as described in (a). The proteins used were (1) phosphorylase a (100000), (2) pyruvate phosphate dikinase, (3) bovine serum albumin (68000), and (4) ovalbumin (43000). (c) Molecular weight of native pyruvate phosphate dikinase. The following proteins were chromatographed on a Bio-Gel A-0.5m column (1 × 59 cm) equilibrated with 50 mM potassium phosphate, 100 mM sodium chloride, and 0.02% sodium azide, pH 7.5: (1) leucine aminopeptidase, 255000; (2) pyruvate phosphate dikinase; (3) bovine serum albumin (68000); (4) ovalbumin (43000); (5) chymotrypsinogen (25000); (6) ribonuclease (13500).

phosphoenolpyruvate via reaction 1c, as described earlier (Milner et al., 1978). In a typical experiment, the reaction mixture contained, in micromoles, imidazole–HCl, 330; $MgCl_2$, 66; NH_4Cl, 167; [^{32}P]phosphoenolpyruvate, 5.4 (7790 cpm/nmol); and enzyme 65.6 mg (sp act. = 12.1) in a final volume of 4.6 mL. The reaction mixture was incubated at 30 °C for 30 min, and then the excess [^{32}P]phosphoenolpyruvate and salts were removed by gel filtration on a Sephadex G-50 (coarse) (1.5 × 25 cm) equilibrated in 0.1 M NH_4HCO_3, pH 7.8, at 4 °C. After determination of the extent of incorporation of radioactivity into the enzyme, the solution was lyophilized.

S-Carboxymethylation and Digestion of the Phosphorylated Enzyme. The phosphoenzyme was S-carboxymethylated in 5 M guanidine hydrochloride as described by Hirs (1967). The salts were then removed by vacuum dialysis against 0.1 M NH_4HCO_3, adjusted to pH 8.6 with NH_4OH, and lyophilized. The lyophilized protein was suspended in 2.0 mL of 0.1 M NH_4HCO_3, pH 7.8, and digested at 37 °C for 90 min with trypsin at a substrate to trypsin ratio of 30:1. During this time the suspension completely clarified.

Amino Acid Analysis. Amino acid analysis was carried out on a Durrum-500 amino acid analyzer following hydrolysis of the peptide samples in constant boiling HCl for 20 h.

Assay of Enzymic Activity. The enzyme was assayed in the direction of ATP formation by the coupled spectrophotometric assay of Milner et al. (1975).

Sequence Determination. The amino acid sequence of the peptide was determined by using the dansyl-Edman procedure (Gray, 1967), and the dansylated amino acids were identified by thin-layer chromatography on polyamide plates (Woods & Wang, 1967; Hartley, 1970). Carboxypeptidase B digestions were carried out in 0.1 M NH_4HCO_3, pH 7.8, for various times, and the liberated amino acids were identified by amino acid analysis.

Results

Isolation of the Phosphohistidyl Peptide. Due to the instability of the phosphohistidyl linkage at acid pH, it was not possible to employ conventional procedures for the isolation

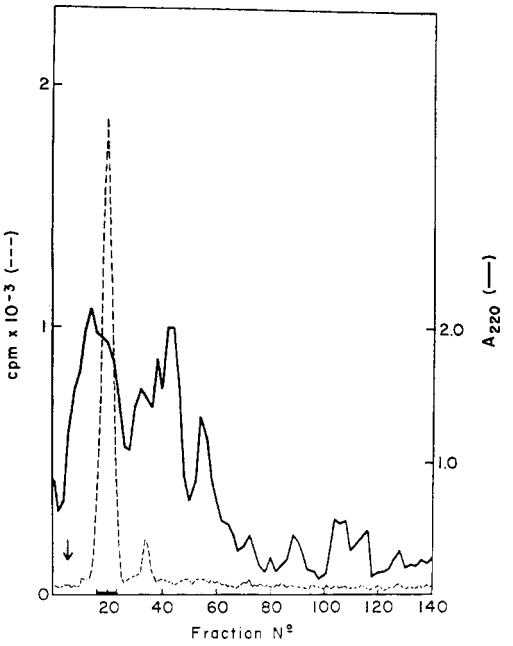

FIGURE 2: First DEAE-cellulose chromatography. Chromatography of the peptides obtained from a tryptic digest of [^{32}P]phosphorylated pyruvate, phosphate dikinase. A linear gradient of ammonium bicarbonate from 5 to 500 mM was initiated at fraction 5 (arrow). Fractions (3.5 mL) containing the desired peptide were pooled as indicated.

of the [^{32}P]phosphohistidyl peptide. However, Wang et al. (1972) and Rose et al. (1975) have demonstrated that phosphohistidyl peptides can be purified by a simple, two-step procedure which takes advantage of the decrease in the cationic character of these peptides following removal of the phosphate moiety at acid pH. The procedure we have employed in this study is, in principle, identical with that successfully developed by these authors.

In one series of experiments, pyruvate phosphate dikinase was phosphorylated, as described under Methods, to a level of 1.33 mol of [^{32}P]orthophosphate/mol of the dimeric enzyme, equivalent to 264.7 nmol of the phosphohistidyl peptide. Following S-carboxymethylation and tryptic digestion, the peptide mixture was applied to a 1.5 × 10 cm column of DEAE-cellulose, equilibrated in 5 mM NH_4HCO_3 at 4 °C, and washed with 2 column volumes of the same buffer. No radioactivity was eluted from the column during this procedure. A 500-mL linear gradient of ammonium bicarbonate from 5 to 500 mM was then applied, and the eluted fractions were analyzed for peptide material at 220 nm and for radioactivity. As can be seen from Figure 2, only one major peak of radioactivity was observed to be eluted from the column. The small peak of radioactivity, which was seen to elute after the major peak in Figure 1, most probably results from incomplete digestion of the protein since it was not observed when the entire peptide isolation procedure was repeated. The fractions containing the major peak of radioactivity were pooled and lyophilized.

The lyophilized material was redissolved in 1.4% pyridine–acetate, pH 1.9, and incubated at 37 °C for 5 h to achieve dephosphorylation of the [^{32}P]phosphohistidine peptide. From the known rate of decay of the phosphohistidine linkage under these conditions (Spronk et al., 1976), complete dephosphorylation of the phosphohistidine peptide will occur in this time. The peptide mixture was then lyophilized, redissolved in 3.0 mL of water, applied to a second DEAE-cellulose column identical with that described earlier, and eluted in the same manner.

Table I: Amino Acid Composition of the Histidyl Peptide[a]

Asx		Met[b]	1.0 (1)
Thr	1.1 (1)[c]	Ile	
Ser	1.1 (1)	Leu	
Glx		Tyr	
Pro		Phe	
Gly	2.1 (2)	Lys	
Ala	3.1 (3)	His	1.0 (1)
Val	1.5 (2)	Arg	1.3 (1)
Cys			

[a] These values are the average of the analysis of two separate 20-h hydrolyses. [b] Determined by hydrolysis in the presence of 0.05% β-mercaptoethanol. [c] Values in parentheses are the number of residues determined from sequence analyses.

Chart I[a]

NH_2-Gly-Gly-Met-Thr-Ser-His-Ala-Ala-Val-Val-Ala-Arg-CO_2H

[a] →, determined by the dansyl Edman procedure; ←, determined by carboxypeptidase B.

As can be seen from Figure 3, a small peak of peptide-containing material was eluted in the 5 mM ammonium bicarbonate wash prior to application of the gradient. This material contained a single fluram-positive spot when chromatographed in butanol–pyridine–acetic acid–water (15:12:3:10 v/v) and contained histidine, as determined by a positive Pauly test. Furthermore, only glycine was present as the N-terminal amino acid, indicating that the material contained only one peptide. Of the six to seven contaminating peptides which eluted with the gradient, none contained histidine, as determined by the Pauly test after thin-layer chromatography as described above. Since previous studies (Spronk et al., 1976) have shown that the phosphate is linked to the histidyl residue, this observation suggests that the dephosphorylation reaction went to completion. In addition, it should be noted that the liberated [^{32}P]orthophosphate comigrates with the contaminating peptides after dephosphorylation.

Due to the low concentration of ammonium bicarbonate at which the phosphorylated histidyl peptide and the accompanying contaminating peptides elute from the first DEAE-cellulose column, it was observed that there was some variation in the fraction number at which these peptides eluted on the second chromatography. In fact, during the purification of the histidyl peptide on a subsequent occasion, some contaminating peptides eluted with the histidyl peptide and a third identical chromatography step was required to remove these contaminants. The histidyl peptides isolated on both occasions were identical.

The amino acid analysis of the purified histidyl peptide is shown in Table I, and the sequence of the dodecapeptide, as determined by the manual dansyl-Edman procedure (Gray, 1967), is given in Chart I. The dansyl amino acids on the carboxyl side of the histidine residue were hydrolyzed for 18 h, rather than the conventional 7 h, in order to obtain more complete cleavage of these acid-resistant amide bonds. Each amino acid in the sequence could, therefore, be unambiguously identified. The valine–valine sequence was also confirmed by the appearance of the dansyl derivative of this dipeptide (Sutton & Bradshaw, 1978). The entire isolation procedure has been repeated and gave identical results with yields of the purified peptide of 11.9% and 9.9%.

Discussion

Pyruvate phosphate dikinase is the only enzyme known to

Table II: Comparison of Phosphohistidine Peptides from Various Sources

pyruvate phosphate dikinase	Gly-Gly-Met-Thr-Ser-*His*-Ala-Ala-Val-Val-Ala-Arg
succinyl-CoA synthetase[a]	– – – Met-Gly-*His*-Ala-Gly-Ala-Ile - Ile - Ala
phosphotransferase[b]	Asp-Glu-Thr-Gly-Ile - *His*-Ala-Arg-Pro-Ala-Thr-Met
phosphoglycerate mutase[c] (yeast)	Leu-Val-Leu-Val-Arg-*His*-Gly-Glu-Ser-Glu-Trp-Asp
phosphoglycerate mutase[d] (chicken)	Leu-Asp-Asp-Glu-Ser-*His*-Arg

[a] Wang et al., 1972. [b] Beyreuther et al., 1977. [c] Han & Rose, 1979. [d] Rose et al., 1975.

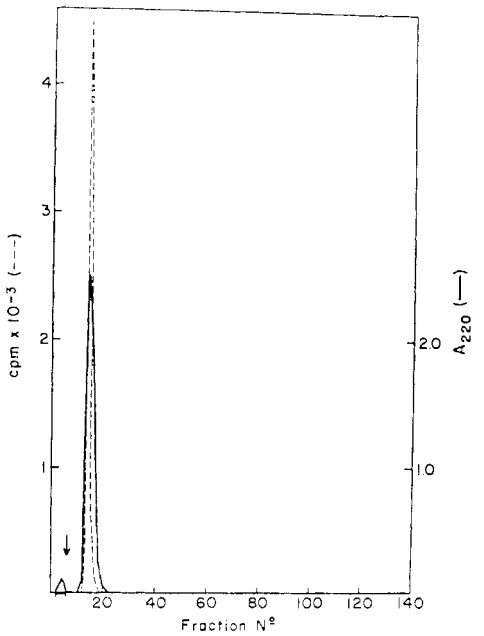

FIGURE 3: Second DEAE-cellulose chromatography. The peptides obtained from the first DEAE-cellulose chromatography were subjected to mild acid treatment (see text) and then rechromatographed under conditions identical with those described in the legend to Figure 2.

contain both enzyme-pyrophosphate and enzyme-phosphate intermediates. It is likely that these phosphate intermediates are linked to the same histidyl residue, although the instability of the histidyl–pyrophosphate linkage has prevented direct demonstration of this feature of the enzyme. The amino acid sequence around this pivotal histidyl residue has several interesting features. First, the appearance of both threonine and serine residues immediately adjacent to the active-site histidyl residue is unusual in that all three of these amino acids are potentially phosphorylatable. The extensive investigations conducted by Spronk et al. (1976) appear to firmly support the view that the histidine is the real acceptor of the phosphate moieties in the enzyme-catalyzed reaction, and there was no indication of phosphorylated seryl or threonyl residues. It is conceivable that one or both of these hydroxylated amino acids may provide part of the binding site for the divalent metal ion required to chelate the phosphate groups during the reaction. Only more detailed studies of the arrangement of other regions of the active site in relation to the histidyl peptide can provide answers to this speculative role.

A second notable feature of the histidyl peptide is the hydrophobic character of the region of the peptide on the carboxyl side of the histidine residue. Table II shows a comparison of the peptide obtained in this study with the sequences of phosphorylated histidyl peptides obtained from a variety of other sources. From this table, it can be seen that succinyl-CoA synthetase also contains a sequence of hydrophobic amino acids on the carboxyl side of the phosphorylated histidyl residue. However, it is apparent that there is little absolute homology between any of these squences and the pyruvate phosphate dikinase peptide.

When the sequence of the histidyl dodecapeptide was analyzed by the empirical method of Chou & Fasman (1974), an α helix was predicted to occur from the threonine residue through to the carboxyl terminal of the peptide. Furthermore, there appears to be a strong potential for the N-terminal portion of the peptide to form a β turn (Chou & Fasman, 1977). Since it is known that both phosphorylation and pyrophosphorylation induce significant changes in the conformation of the enzyme (Michaels et al., 1978), it is possible that these predicted structures may change significnatly during the enzyme-ctalyzed reaction.

Finally, the occurrence of a methionine residue in the histidyl peptide provides an opportunity to determined the sequence beyone the carboxy terminal arginine residue found in this present study. In doing so, it is possible that we may encounter either of the peptides modified by bromopyruvate (Yoshida & Wood, 1978) or the 2,3-dialdehyde derivative of AMP (Evans et al., 1980). The isolation and sequence of these two affinity-labeled peptides is at present under investigation in this laboratory.

Acknowledgments

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Pyruvate Phosphate Dikinase: Affinity Labeling of the Adenosine 5′-Triphosphate–Adenosine 5′-Monophosphate Site†

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ABSTRACT: The 2′,3′-dialdehyde of adenosine 5′-phosphate (oAMP) is shown to specifically modify the adenosine 5′-triphosphate (ATP)–AMP site of pyruvate phosphate dikinase from *Bacteroides symbiosus*, and in the presence of sodium cyanoborohydride the enzyme is irreversibly inactivated. The overall dikinase reaction involves three partial reactions, each of which is catalyzed by a functionally independent site; one site catalyzes the ATP–AMP exchange, the second the P_i–PP_i exchange, and the third the pyruvate–phosphoenolpyruvate exchange. The specificity of oAMP for the ATP–AMP site was demonstrated by examining the effect of inactivation on the three exchange reactions. When enzyme was incubated in the presence of oAMP and reducing agent, the ATP–AMP exchange was completely inhibited, the P_i–PP_i exchange was inhibited 15%, and the pyruvate–phosphoenolpyruvate exchange was not affected, showing that oAMP specifically inhibited the ATP–AMP site. The weak inhibition of the P_i–PP_i exchange may be because of its close proximity to the ATP–AMP site. In addition, oAMP fit the criteria of an affinity label. The compound is a competitive inhibitor with respect to AMP. The rate of inactivation of the enzyme followed pseudo-first-order kinetics, and a plot of the rate of inactivation vs. oAMP concentration gave a typical saturation curve. These results are consistent with a two-step reaction in which oAMP is rapidly and specifically bound to the ATP–AMP site and then a Schiff's base is formed inactivating the enzyme. AMP completely protected against the inactivation at concentrations >1.0 mM. Inorganic phosphate did not protect, while 1.0 mM pyruvate protected somewhat, against inactivation of the dikinase. Binding studies with [U-^{14}C]oAMP showed that there was 50% inactivation when 1 mol of the analogue was bound per mol of dimeric enzyme. At higher concentrations of oAMP, the inactivation became nonlinear as though modification of the first nucleotide site decreased the reactivity on the second site for oAMP but left it enzymatically active. These results are in contrast to previous experiments with bromopyruvate which reacted at the pyruvate–phosphoenolpyruvate site and gave complete inactivation of the enzyme when one site was modified, so suggesting half-site reactivity.

Pyruvate phosphate dikinase (EC 2.7.9.1) catalyzes the reversible phosphorylation of pyruvate and inorganic phosphate by utilizing the β and γ phosphates of ATP.[1]

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{phosphoenolpyruvate} + \text{AMP} + PP_i \quad (1)$$

Several lines of evidence support the conclusion that the overall reaction catalyzed by enzyme preparations from *Bacteroides symbiosus* and *Propionibacterium shermanii* involves three partial reactions.

$$E + \text{ATP} \rightleftharpoons E\text{–PP} + \text{AMP} \quad (1a)$$

$$E\text{–PP} + P_i \rightleftharpoons E\text{–P} + PP_i \quad (1b)$$

$$E\text{–P} + \text{pyruvate} \rightleftharpoons E + \text{phosphoenolpyruvate} \quad (1c)$$

Equilibrium exchange studies (Evans & Wood, 1968; Milner & Wood, 1972; Milner & Wood, 1976), initial velocity steady-state kinetics (Milner & Wood, 1972, 1976), and product inhibition patterns (Milner & Wood, 1972) are consistent with a three-site tri-uni-uni ping-pong mechanism. The presence of phosphoryl- and pyrophosphoryl-enzyme forms has been demonstrated (Milner & Wood, 1976; Spronk et al., 1976). According to these observations, three functionally distinct sites catalyze, first, the ATP–AMP exchange, second, the P_i–PP_i exchange, and, third, the pyruvate–phosphoenolpyruvate exchange. Additionally, a histidyl residue is located so that it serves as a phosphoryl and pyrophosphoryl group carrier between these sites (Milner et al., 1978; Yoshida & Wood, 1978).

The present work was undertaken to further investigate the structural relationships among the three substrate sites by covalently modifying the ATP–AMP site with a chemically reactive substrate analogue of AMP. Inactivation experiments have shown that the 2′,3′-dialdehyde derivative of AMP (oAMP)[1] specifically modifies the ATP–AMP site of the dikinase. Several investigators have used dialdehyde derivatives of ATP, ADP, and UDP as site-specific covalent mod-

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[1] Abbreviations used: AMP, adenosine 5′-phosphate; oAMP, 2′,3′-dialdehyde AMP; ATTP, adenosine 5′-tetraphosphate; Cl_3AcOH, trichloroacetic acid; $NaCNBH_4$, sodium cyanoborohydride; ATP, adenosine 5′-triphosphate; E-P, phophoryl-enzyme intermediate; E-PP, pyrophosphoryl-enzyme intermediate; NADH, reduced nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; GMP, guanosine 5′-phosphate; IMP, inosine 5′-phosphate.

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