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Modification of Pyruvate, Phosphate Dikinase with Pyridoxal 5′-Phosphate: Evidence for a Catalytically Critical Lysine Residue†

Nelson F. B. Phillips, Neil H. Goss, and Harland G. Wood*

ABSTRACT: Pyruvate, phosphate dikinase from *Bacteroides symbiosus* is strongly inhibited by low concentrations of pyridoxal 5′-phosphate. The inactivation follows pseudo-first-order kinetics over an inhibitor concentration range of 0.1–2 mM. The inactivation is highly specific since pyridoxine and pyridoxamine 5′-phosphate, analogues of pyridoxal 5′-phosphate, which lack an aldehyde group, caused little or no inhibition even at high concentrations. The unreduced dikinase–pyridoxal 5′-phosphate complex displays an absorption maxima near 420 nm, typical for Schiff base formation. Following reduction of the Schiff base with sodium borohydride, N^6-pyridoxyllysine was identified in the acid hydrolysate. When the enzyme was incubated in the presence of pyridoxal 5′-phosphate and reducing agent, the ATP/AMP, P_i/PP_i, and pyruvate/phosphoenolpyruvate isotopic exchange reactions were inhibited to approximately the same extent, suggesting that the modification of the lysyl moiety causes changes in the enzyme that affect the reactivity of the pivotal histidyl residue. Phosphorylation of the histidyl group appears to prevent the inhibitor from attacking the lysine residue. On the other hand, addition of pyridoxal 5′-phosphate to the pyrophosphorylated enzyme promotes release of the pyrophosphate and yields the free enzyme which is subject to inhibition.

Pyruvate, phosphate dikinase (EC 2.7.9.1) catalyzes the reversible formation of phosphoenolpyruvate from pyruvate, a reaction which is dependent on ATP and inorganic phosphate.

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

The overall reaction catalyzed by enzyme preparations from *Bacteroides symbiosus* and *Propionibacterium shermanii* in-

† From the Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106. *Received November 19, 1982.* Supported by Grant PCM 80-22701 from the National Science Foundation.

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volves three partial reactions, each of which is catalyzed at a distinct subsite.

$$\text{Enz} + \text{ATP} \rightleftharpoons \text{Enz}\text{–}{}^{\beta}\text{P}^{\gamma}\text{P} + \text{AMP} \quad (1a)$$

$$\text{Enz}\text{–}{}^{\beta}\text{P}^{\gamma}\text{P} + \text{P}_i \rightleftharpoons \text{enz}\text{–}{}^{\beta}\text{P} + \text{P}^{\gamma}\text{P}_i \quad (1b)$$

$$\text{Enz}\text{–}{}^{\beta}\text{P} + \text{pyruvate} \rightleftharpoons \text{Enz} + [{}^{\beta}\text{P}]\text{phosphoenolpyruvate} \quad (1c)$$

Evidence in support of this conclusion 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). Additional evidence was obtained by the demonstration of a phosphoryl and pyrophosphoryl form of the enzyme (Milner & Wood, 1976; Spronk et al., 1976). Finally, it has been shown that the phosphoryl moiety is bound to the enzyme through a phosphoramidate linkage to the 3′-nitrogen of a histidine residue (Spronk et al., 1976). This histidyl residue is considered to be centrally located in the enzyme active site, surrounded by the three independent partial reaction subsites, and serves as a phosphoryl and pyrophosphoryl group carrier between these subsites (Milner et al., 1978; Yoshida & Wood, 1978).

Although it has been established that the three subsites are catalytically independent, several lines of evidence have indicated that these subsites may be more closely associated than was previously realized. Chemical modification of the ATP/AMP subsite with the 2′,3′-dialdehyde derivative of AMP (oAMP)[1] completely inhibited the ATP/AMP isotope exchange, while the pyruvate/P-enolpyruvate exchange was unaffected, but the P_i/PP_i exchange was inhibited 15% (Evans et al., 1980). Furthermore, pyruvate, a substrate of partial reaction 1c, protected 30% of the enzyme activity from inactivation by oAMP. In other studies, when the pyruvate/P-enolpyruvate subsite was modified with bromopyruvate, the pyruvate/P-enolpyruvate exchange was completely inhibited while the ATP/AMP exchange was unaffected. The P_i/PP_i exchange, however, was inhibited to 50% (Yoshida & Wood, 1978). These observations have led to the suggestion that the P_i/PP_i subsite may be centrally located and that alteration of either of the other subsites affects its functions.

PLP has been shown to selectively modify primary amino groups in or near phosphate binding sites of many proteins (Rippa et al., 1967; Colombo & Marcus, 1974). The present work focuses on the modification of the enzyme with PLP and constitutes a further investigation of the structural relationships among the three subsites of this unusual enzyme.

Experimental Procedures

Materials

[^{32}P]Orthophosphate, [U-^{14}C]AMP, and [2-^{14}C]pyruvate were obtained from New England Nuclear; phosphoenolpyruvate and NADH were from P-L Biochemicals, Inc.; pyruvate, pyridoxal 5′-phosphate, nucleotides, sodium pyrophosphate, pyridoxal, pyridoxine, pyridoxamine 5′-phosphate, and sodium borohydride were from Sigma Chemical Co.; poly(L-lysine) (M_r 3000) was from United States Biochemical Corp. and lactate dehydrogenase from Boehringer Mannheim. All other chemicals were of analytical grade.

Methods

Purification and Assay of Pyruvate, Phosphate Dikinase. Pyruvate, phosphate dikinase was prepared from *B. symbiosus* as described by Goss et al. (1980). The enzyme was assayed either by measuring the rate of pyruvate formation from P-enolpyruvate, by coupling with lactate dehydrogenase, or by the radiochemical assay in the direction of P-enolpyruvate formation by measuring the formation of radioactive PP_i from [^{32}P]P_i (Milner et al., 1975).

Isotope Exchange Assays. The ATP/AMP, P_i/PP_2, and pyruvate/P-enolpyruvate isotopic exchanges were assayed essentially as described by Evans et al. (1980). The rate of all isotopic exchange reactions were calculated by the method of Wahl & Bonner (1951).

Preparation of N^6-Pyridoxyllysine. Poly(N^6-phosphopyridoxyllysine) was prepared by the method described by Schnackerz & Noltmann (1971) for poly(N^6-pyridoxyllysine) except that pyridoxal 5′-phosphate was substituted for pyridoxal. Poly(L-lysine hydrobromide) (M_r 3000; 20 mg) was treated with pyridoxal 5′-phosphate (0.1 mmol) in 50 mM sodium acetate (pH 6.0). The reaction mixture (1 mL) was allowed to stand for 15 min at 0 °C and was then reduced with sodium borohydride (1 mmol). After a further incubation of 15 min, low molecular weight reactants were removed by dialysis against distilled water at 4 °C for 24 h. The dialysate was lyophilized, and N^6-pyridoxyllysine was prepared by acid hydrolysis in 6 M HCl at 110 °C in vacuo for 20 h. During the entire procedure, the sample was exposed to light as little as possible, since PLP is sensitive to light (Ritchey et al., 1977).

Inactivation of Pyruvate, Phosphate Dikinase by Pyridoxal 5′-Phosphate. Inactivation studies were carried out under the conditions given in the legends of Figures 2 and 3.

Modification with Pyridoxal 5′-Phosphate for Determination of Stoichiometry. Pyruvate, phosphate dikinase (54 μg) having a specific activity of 11 units mg^{-1} was incubated in 60 mM imidazole hydrochloride buffer (pH 6.8) containing 20 mM NH_4Cl, 20 mM $MgCl_2$, and varying concentrations of PLP in a final volume of 250 μL. The modification was performed in the dark for 15 min at 30 °C and then was reduced with $NaBH_4$ in a 10-fold molar excess over PLP. The reaction solutions were incubated for a further 10 min. The reduced samples were desalted by the rapid microcentrifuge desalting technique as described by Helmerhorst & Stokes (1980). A 2-mL syringe containing Sephadex G-25 (fine), preequilibrated with 0.1 M imidazole hydrochloride (pH 6.8) was used for this purpose. Aliquots of the desalted solutions were then withdrawn for determination of enzyme activity, and absorbancy was measured at 325 nm. The quantity of PLP bound to the protein was estimated by assuming a value of 10 150 cm^{-1} M^{-1} for the extinction coefficient at 325 nm (Fischer et al., 1963). For all calculations, a molecular weight of 188 000 was assumed for dimeric pyruvate, phosphate dikinase (Goss et al., 1980).

Identification of the Modified Residue. Pyruvate, phosphate dikinase (0.5 mg) was reacted with PLP as described above. After reduction with $NaBH_4$, the modified enzyme was extensively dialyzed in the dark at 4 °C against 50 mM sodium acetate, pH 6, followed by dialysis against distilled water for 24 h. The dialyzed solution was lyophilized and hydrolyzed in 6 N HCl at 110 °C in vacuo for 20 h. The hydrolysate was lyophilized after diluting with distilled water. To elimate residual HCl, the sample was dried over NaOH pellets in a heated (60 °C) vacuum desiccator. The sample was dissolved in 50% pyridine, and aliquots were applied to thin-layer cel-

[1] Abbreviations: E∼P, phosphoryl-enzyme intermediate; E∼PP, pyrophosphoryl-enzyme intermediate; oAMP, 2′,3′-dialdehyde derivative of AMP; PEP (P-enolpyruvate), phosphoenolpyruvate; PLP, pyridoxal 5′-phosphate.

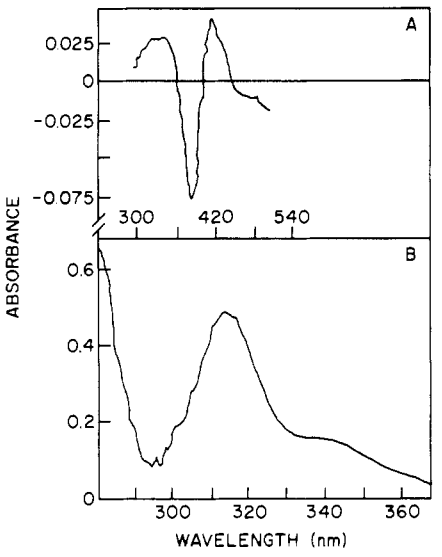

FIGURE 1: Difference spectrum of the pyruvate, phosphate dikinase–pyridoxal 5′-phosphate complex. Spectral measurements were made with a cylindrical dual cuvette on a Gilford 250 spectrophotometer. One compartment of the cuvette contained 0.16 unit of pyruvate, phosphate dikinase, 0.5 mM PLP, 20 mM $MgCl_2$, and 20 mM NH_4Cl in 60 mM imidazole hydrochloride (pH 6.8) in a final volume of 1 mL. In the other compartment, enzyme was omitted. (A) The difference spectrum obtained after an incubation time of 20 min by running a wavelength scan over the range indicated. (B) Spectrum after treatment with sodium borohydride. The reaction conditions were as described above except that after the mixture was incubated for 20 min, $NaBH_4$ was added to give a final concentration of 5 mM.

lulose plates and subjected to (a) electrophoresis at 500 V for 60 min in pyridine–acetic acid–H_2O (10:0.5:89.5 v/v), pH 6.5, or (b) ascending chromatography in 1-butanol–acetic acid–pyridine–water (15:3:10:12 v/v). After the cellulose plates were dried, a blue fluorescent spot with a mobility identical with that of synthetic N^6-pyridoxyllysine was visible under ultraviolet light.

Results

Difference Spectrum of the PLP–Pyruvate, Phosphate Dikinase Complex. When pyruvate, phosphate dikinase was reacted with PLP in imidazole hydrochloride (pH 6.8), the difference spectrum showed characteristic changes typical for this type of protein–ligand interaction (Figure 1A). The absorption in the 420-nm region has been found in all PLP–protein complexes and is attributed to the formation of a Schiff base (Metzler, 1957). The smaller absorption peak at 340 nm suggests that PLP may have additional interactions with the enzyme. The spectrum shown was recorded 20 min after the addition of PLP to the enzyme. We have observed that this difference spectrum undergoes modification with time; the peak at 340 nm was less pronounced at shorter incubation times; with longer incubation times, the peak at 420 nm was found to decrease, and the trough at 380 nm assumes a more negative value. Similar observations have been made when PLP interacts with 6-phosphogluconic dehydrogenase (Rippa et al., 1967). When the PLP–enzyme complex was reduced with sodium borohydrate, the spectrum showed a new absorption maxima at 315 nm (Figure 1B). These data are consistent with Schiff base formation at a primary amino group on the enzyme which is reducible by sodium borohydride.

Inactivation of Pyruvate, Phosphate Dikinase by Pyridoxal 5′-Phosphate. Treatment of pyruvate, phosphate dikinase (44 μg) with PLP (0.5 mM) results in a time-dependent loss of dikinase activity. The inactivation is pseudo first order over

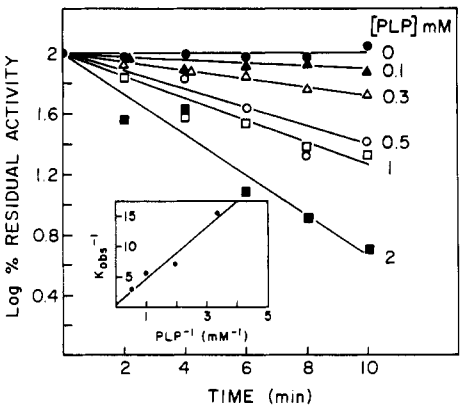

FIGURE 2: Inactivation of pyruvate, phosphate dikinase by pyridoxal 5′-phosphate as a function of time. The reaction mixture contained 60 mM imidazole hydrochloride (pH 6.8), 20 mM $MgCl_2$, 20 mM NH_4Cl, 14 μg of enzyme, and PLP as indicated in a final volume of 250 μL. Incubation was at 30 °C in the dark. At the times shown, aliquots of the reaction mixture were assayed for residual activity by the spectrophotometric assay. (Inset) The observed first-order rate constant, K_{obsd}, as a function of the reciprocal of PLP concentration. The K_{obsd} was calculated from $t_{1/2}$ as described in the text, and the straight line drawn represents a least-squares fit of data points.

Table I: Effect of Pyridoxal 5′-Phosphate Analogues on Enzyme Activity[a]

inhibitor	concentration of inhibitor (mM)	% residual enzyme activity
none		100
pyridoxal 5′-phosphate	0.5	45
	1	34
pyridoxal	1	107
	10	107
pyridoxine	1	80
	10	91
pyridoxamine 5′-phosphate	10	75

[a] Samples of enzyme (14 μg) were incubated for 15 min at 30 °C in reaction mixtures (250 μL) containing 60 mM imidazole hydrochloride (pH 6.8), 20 mM $MgCl_2$, 20 mM NH_4Cl, and the compounds and their concentrations as indicated. Aliquots were removed and analyzed for enzymic activity by the spectrophotometric assays, 10 min after the addition of a 10-fold molar excess of $NaBH_4$.

a concentration range of 0.1–2 mM PLP (Figure 2). The inactivation half-times, $t_{1/2}$, were calculated by determining the time required for PLP to cause a 50% inhibition of dikinase activity. The half-life for inactivation is related to the observed first-order rate constant (K_{obsd}) by the expression $K_{obsd} = 0.693/t_{1/2}$. As shown in the inset of Figure 2, a plot of $1/K_{obsd}$ vs. $1/[PLP]$ is a straight line with a finite vertical intercept giving a limiting rate constant for the inactivation of 0.034 s^{-1}. The dissociation constant, K_I, was determined from the horizontal intercept and gave a value of 6.7 mM (Plapp, 1982). Thus, the inactivation shows saturation kinetics and provides evidence that PLP forms a dissociable complex prior to Schiff's base formation.

Specificity of PLP-Mediated Dikinase Inactivation. In order to assess the specificity of PLP for the active site of pyruvate, phosphate dikinase, analogues of PLP were tested for their effect on the enzyme activity. As seen from Table I, pyridoxine, pyridoxamine 5′-phosphate, which lacks an aldehyde group, and pyridoxal, which has an aldehyde group but lacks a phosphate group, caused little or no inhibition when tested under the experimental conditions used for PLP. The interpretation of the result obtained with pyridoxal is com-

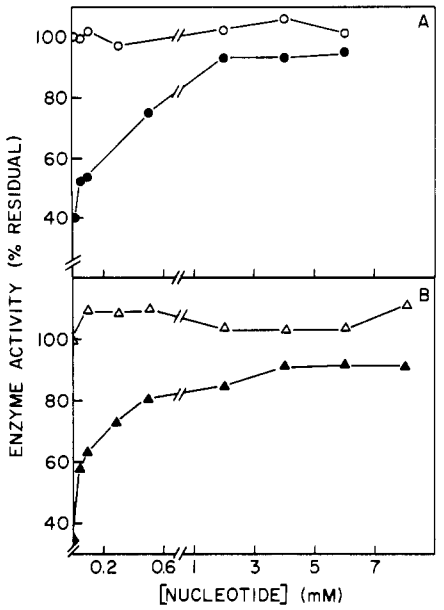

FIGURE 3: Protection of enzyme against inactivation by PLP: (A) by ATP and (B) by AMP. Samples of pyruvate, phosphate dikinase (14 μg) were incubated in 60 mM imidazole hydrochloride, 20 mM NH_4Cl, 0.5 mM PLP, and increasing concentrations of ATP (●) or AMP (▲) as indicated in a final volume of 250 μL. $MgCl_2$ (20 mM) was included when AMP was the protectant. The control reaction mixture was as above except that only ATP (○) or AMP (△) were included without PLP. After 15 min at 30 °C, a 10-fold molar excess of $NaBH_4$ was added, and the solution was incubated for a further 10 min after which aliquots were assayed for residual enzyme activity by the spectrophotometric assay when AMP was the protectant and the radiochemical assay when ATP was the protectant.

plicated by the fact that in acid and neutral solutions, most of the pyridoxal is present in its hemiacetal form (Heyl et al., 1951).

Protection of the Enzyme from Inactivation by PLP. One important criterion to ascertain whether an inhibitor is active site directed is to observe protection by the normal substrate against the inactivation process. The effect of several compounds related to the reactions catalyzed by pyruvate, phosphate dikinase on the inactivation of the enzyme by PLP was tested. The substrate ATP affords almost complete protection against inhibition (Figure 3A). This protection is not due to the formation of E~PP, since the experiment was done in the absence of Mg^{2+}, which is essential for the formation of E~PP. The product of the first partial reaction, AMP (eq 1a), also affords significant protection (Figure 3B). The substrate (inorganic phosphate) and product (pyrophosphate) of the second partial reaction (eq 1b) did not protect against this inactivation when present at concentrations up to 9 mM (data not shown). This implies that the modification occurs at a portion of the active site which is not involved in the binding of these compounds.

Phosphoenolpyruvate does not protect against this inactivation in the absence of $MgCl_2$ (Figure 4A). However, when added together with Mg^{2+}, P-enolpyruvate afforded almost complete protection (Figure 4B). It has been shown previously that Mg^{2+} is required for the phosphoryl group transfer from P-enolpyruvate to the histidine moiety of the enzyme (Moskovitz & Wood, 1978). Hence, the results indicate that the binding of P-enolpyruvate per se does not exclude PLP from interacting with the enzyme, but when the enzyme is phosphorylated, PLP does not interact with the critical lysine residue. To investigate this possibility, the phosphorylated form of the enzyme was prepared and isolated as described in the legend of Figure 5. The isolated E~P was reacted with PLP,

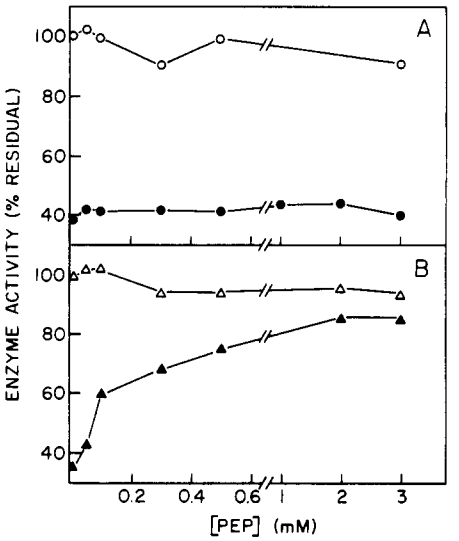

FIGURE 4: Effect of P-enolpyruvate on the inhibition of pyruvate, phosphate dikinase by pyridoxal 5′-phosphate. Samples of the enzyme (14 μg) were reacted with PLP under the reaction conditions as described in Figure 3 except that instead of nucleotide, varying concentrations of PEP were used. (A) PEP with no $MgCl_2$ (●). (B) PEP and 20 mM $MgCl_2$ (▲). The control reaction mixture was as above except that only PEP (○) or PEP plus $MgCl_2$ (△) was included without PLP. The residual enzyme activity was determined by the spectrophotometric assay.

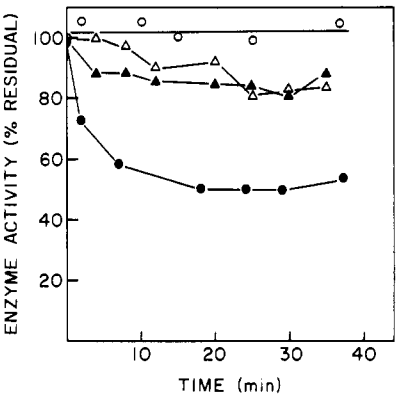

FIGURE 5: Effect of pyridoxal 5′-phosphate on the E~P or E~PP forms of pyruvate, phosphate dikinase. E~P was prepared by incubating 54 μg of the dikinase in 60 mM imidazole hydrochloride (pH 6.8) buffer containing 12 mM $MgCl_2$, 8 mM NH_4Cl, and 0.5 mM PEP in a final volume of 250 μL. The E~PP was prepared in the same buffer as above, except that 4 mM $MgCl_2$ was used and 0.5 mM ATP was included instead of PEP. After 10 min at 25 °C, the E~P and E~PP were purified by the rapid microcentrifuge desalting technique (Helmerhorst & Stokes, 1980). The isolated E~P (▲) and E~PP (●) were reacted with 0.5 mM PLP in a reaction buffer containing 60 mM imidazole (pH 6.8), 20 mM $MgCl_2$, and 20 mM NH_4Cl at 25 °C. At the times indicated, the residual enzyme activity was determined by the spectrophotometric assay. In the controls, the E~P (△) and the E~PP (○) were reacted in the same buffer in the absence of PLP.

and as seen from Figure 5, PLP caused little or no inhibition of this form of the enzyme. However, the pyrophosphorylated form of the enzyme, prepared as described in the figure legends, was found to be inactivated by PLP (Figure 5). In contrast to E~P, it was found with E~PP that PLP caused the breakdown of E~PP to PP_i and free enzyme. This was determined by treating E~P[^{32}P]P (prepared by using [γ-^{32}P]ATP) with PLP for 15 min at 25 °C, after which an aliquot of the reaction solution was applied to poly(ethylenimine) thin-layer plates. The chromatograms were developed in 0.8 M NH_4HCO_3. There was 40% more radioactivity in the region where PP_i migrates (R_f 0.41) in the reaction in the

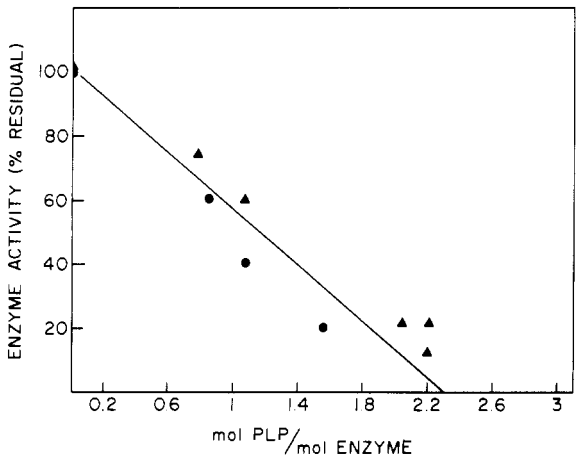

FIGURE 6: Stoichiometry of inactivation of pyruvate, phosphate dikinase by modification with pyridoxal 5′-phosphate. The enzyme samples (54 μg) were incubated with increasing concentrations of PLP and reduced with $NaBH_4$ as described under Methods. A value of 100% initial activity was given to a control enzyme treated with $NaBH_4$ in the absence of PLP. Enzyme activity was determined by the spectrophotmetric assay while the amount of PLP bound to the enzyme was estimated by assuming a value of 10 150 cm^{-1} M^{-1} for the extinction coefficient at 325 nm. (●) and (▲) indicate the results obtained from two separate experiments.

presence of PLP relative to a control in which E∼P[^{32}P]P was incubated in the imidazole buffer in the absence of PLP. Thus, PLP stimulates the hydrolysis of E∼PP, liberating PP_i and free enzyme, which is subsequently inactivated by the PLP.

Equilibrium Exchange Reactions of Modified Enzyme. Pyruvate, phosphate dikinase catalyzes three isotopic exchange reactions at functionally distinct sites: (a) [^{14}C]AMP/ATP; (b) [^{32}P]P_i/PP_i and (c) [^{14}C]pyruvate/P-enolpyruvate. Exchanges a and c were found to be independent of other substrates, and exchange b was dependent on the presence of either P-enolpyruvate or ATP (Milner et al., 1978). To examine the effect of PLP modification of the enzyme on each of these exchange reactions, 1.3 units of enzyme (specific activity 11 units mg^{-1}) was modified with 0.5 mM PLP in 50 μL of 60 mM imidazole hydrochloride (pH 6.8) buffer containing 20 mM NH_4Cl and 4 mM $MgCl_2$. After 15 min at 30 °C, $NaBH_4$ was added in a 10-fold molar excess over PLP. The reaction solution was incubated for a further 10 min, after which the modified enzyme was desalted as described by Helmerhorst & Stokes (1980). A 36-μg sample of the modified enzyme was then used to initiate the exchange reactions which were monitored as described by Evans et al. (1980). The amount of these exchanges occurring in the modified enzyme was calculated and expressed as the precent of exchange activity relative to that catalyzed by the enzyme treated with $NaBH_4$ but in the absence of PLP. The modification of the dikinase by PLP results in a loss of all three exchange activities to approximately the same extent; i.e., the ATP/AMP exchange was inhibited by 68%, the P_i/PP_i exchange by 82%, and the pyruvate/PEP exchange by 86%.

Stoichiometry of the PLP Modification of Pyruvate, Phosphate Dikinase. To determine the relationship between the moles of PLP bound and the loss of enzymatic activity, pyruvate, phosphate dikinase was modified with different concentrations of PLP and reduced as described under Methods. When the residual enzyme activity is plotted as a function of moles of PLP incorporated based on the absorption at 325 nm, there was a linear relationship to 80% inactivation (Figure 6). Extrapolation to 100% loss of activity gave a mean of 2.3 ± 0.3 mol of PLP bound/mol of enzyme in two separate experiments.

Isolation and Identification of N^6-Pyridoxyllysine from the Reduced PLP–Pyruvate, Phosphate Dikinase Complex. The reduced enzyme–PLP complex was dialyzed exhaustively against 50 mM sodium acetate (pH 6) at 4 °C followed by dialyses against distilled water. The dialyzed solution was then lyophilized and hydrolyzed with HCl, as described under Methods. N^6-Pyridoxyllysine was then identified in the hydrolysate by means of chromatography and electrophoresis on cellulose thin-layer plates. Following chromatography, the hydrolysate of PLP–enzyme showed one major fluorescent band under ultraviolet light with an R_f of 0.32 and comigrated with the authentic N^6-pyridoxyllysine. Similarly, in the electrophoresis system, the fluorescent band from the PLP–enzyme hydrolysate comigrated with the N^6-pyridoxyllysine. The relative migration values were +0.47 and +0.5, respectively (expressed in relation to the mobility of the dye, xylene cyanol, taken as −1). The bluish fluorescent bands could be stained with ninhydrin, confirming the presence of a free amino group. A faint fluorescence spot appeared in the electrophoretic system in both the hydrolysate of the enzyme–PLP complex and the synthetic N^6-pyridoxyllysine, the nature of which is unknown.

Discussion

These studies were undertaken to determine if PLP is an active-site-directed inhibitor of the *B. symbiosus*, pyruvate, phosphate dikinase. The findings indicate that a critical lysine residue is modified by PLP. Evidence for this comes from the observation that (i) the inactivation of the enzyme was stoichiometric with the amount of PLP bound and extrapolated to ∼2 mol bound/mol of dimeric enzyme, (ii) the rate of inactivation is pseudo first order and exhibits saturation kinetics, and (iii) pyridoxal phosphate forms a Schiff base with internal hydrogen bonding to a lysyl residue of the dikinase. This is indicated by the absorption peak at 420 nm of the PLP–dikinase complex, the spectral changes giving a new absorption maxima at 315 nm upon addition of $NaBH_4$, and the identification of N^6-pyridoxyllysine in the acid hydrolysate. However, the smaller absorption peak at 340 nm suggests that PLP may have additional interactions with the dikinase. Proposals concerning the structure of the chromophore which absorbs at 340 nm include formation of a Schiff base in a hydrophobic environment, a carbinolamine, or a *gem*-diamine or similar adduct with a second nucleophilic amino acid residue in rapid equilibrium with the Schiff base (Whitman & Tabita, 1978, and references therein). None of these possibilities can be eliminated in the present work.

Previous studies on the interaction of oAMP with the dikinase (Evans et al., 1980) have indicated that oAMP modifies a lysine residue at or near the ATP/AMP binding site. However, the inactivation kinetics shown by oAMP are quite different from that observed with PLP. With [^{14}C]oAMP, the stoichiometric inactivation of the dikinase was not linear after ∼50% inhibition, suggesting that the modification of the first nucleotide site on one subunit decreased the reactivity of the site on the second subunit. In contrast, there was a linear relationship to ∼80% inactivation when the enzyme was modified with PLP. In addition, unlike the inactivation by oAMP which predominantly inhibits the ATP/AMP isotopic exchange reaction, marginally affects the P_i/PP_i exchange, and does not affect the pyruvate/P-enolpyruvate exchange, modification by PLP inhibits all three isotopic exchange reactions to approximately the same extent. Furthermore, the inactivation of the enzyme with PLP is noncompetitive with AMP (data not shown), whereas Evans et al. (1980) demonstrated that oAMP was competitive with AMP. These findings

suggest that the modification of the critical lysyl residue by PLP is not the same as that modified by oAMP. PLP apparently reacts with a lysyl group which affects the reactivity of the pivotal histidyl moiety. Since the histidyl group serves as a phosphoryl and pyrophosphoryl group carrier between the three subsites, this could account for inhibition of all three partial reactions by PLP.

It seems likely that there are changes which occur in the enzyme when the histidyl moiety is phosphorylated which makes the lysyl residue inaccessible to PLP. Support for this conclusion comes from the observation that phosphorylation of the enzyme protects the dikinase from inactivation by PLP. This observation is in accord with those of Yoshida & Wood (1978), who found that phosphorylation and pyrophosphorylation protect the dikinase from inactivation by bromopyruvate, an affinity label of the pyruvate/P-enolpyruvate binding site. However, pyrophosphorylation does not protect against inactivation by PLP. In contrast to the E∼P form, there is an enhancement of the breakdown of E∼PP to PP_i and free enzyme. This enhancement was quite unexpected, and we have no explanation for it. It is known that the E∼PP is less stable than the E∼P (Milner et al., 1978), and PLP may in some way render the E∼PP more unstable. Nevertheless, it does account for the difference in susceptibility of the two forms of the enzymes since the susceptible free form of the enzyme is formed when E∼PP is treated with PLP.

Work is currently being undertaken in this laboratory to identify the lysyl residues modified by oAMP and PLP.

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Registry No. PLP, 54-47-7; PEP, 138-08-9; ATP, 56-65-5; 5′-AMP, 61-19-8; pyruvate, phosphate dikinase, 9027-40-1; L-lysine, 56-87-1.

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