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Identification of Critical Lysyl Residues in the Pyrophosphate-Dependent Phosphofructo-1-kinase of *Propionibacterium freudenreichii*†

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ABSTRACT: Pyrophosphate-dependent 6-phosphofructo-1-kinase (PP_i-PFK) from *Propionibacterium freudenreichii* was inactivated by low concentrations of the lysine-specific reagent pyridoxal phosphate (PLP) after sodium borohydride reduction. The substrates fructose 6-phosphate and fructose 1,6-bisphosphate protected against inactivation whereas inorganic pyrophosphate had little effect. An HPLC profile of a tryptic digest of PP_i-PFK modified at low concentrations of PLP showed a single major peak with only a small number of minor peaks. The major peak peptide was isolated and sequenced to obtain IGAGXTMVQK, where X represents a modified lysine residue, corresponding to Lys-315. Lys-315 was protected from reaction with PLP by fructose 1,6-bisphosphate. As indicated by HPLC maps of PP_i-PFK modified with varying concentrations of PLP, a direct correlation was observed between activity loss and the modification of Lys-315. Two of the minor peptide peaks were shown to contain Lys-80 and Lys-85, which were modified in a mutually exclusive manner. Partial protection against modification of these two residues was provided by $MgPP_i$. The data were used to adjust the sequence alignment of the *Propionibacterium* enzyme with that of ATP-dependent PFK of *Escherichia coli* to identify homologous residues in the substrate binding site. It is suggested that Lys-315 interacts with the 6-phosphate of fructose 6-phosphate and that Lys-80 and -85 may be located near the pyrophosphate binding site.

Recently, this laboratory described the amino acid sequence of the pyrophosphate-dependent phosphofructokinase (PP_i-PFK)[1] from *Propionibacterium freudenreichii* (Ladror et al., 1991). The properties of this enzyme differ substantially from the major family of ATP-dependent phosphofructokinases in that it is dimeric as opposed to being a tetramer and that it displays no allosteric properties. On the other hand, a low but significant level of sequence identity with *Escherichia coli* PFK was established to indicate that the PP_i-dependent and the ATP-dependent enzymes are homologous (Ladror et al., 1991). While the overall identity was only 23%, the alignment of the amino-terminal half of PP_i-PFK showed 30% identity, with a much lower similarity in the carboxyl-terminal half. In addition, seven residues which have been shown to be involved in the binding of the sugar phosphate substrate by the ATP-dependent *E. coli* enzyme (Shirakihara & Evans, 1988) could be readily aligned with identical residues in the amino-terminal half of the PP_i-dependent enzyme. Three basic residues found

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[1] Abbreviations: PFK, 6-phosphofructo-1-kinase; Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; DTT, dithiothreitol; PP_i, inorganic pyrophosphate; PLP, pyridoxal phosphate.

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in the carboxyl half of the ATP-dependent enzyme and involved in the binding of the 6-PO_4 of the substrate could not be aligned with the PP_i-dependent enzyme. The current study was designed to locate additional residues of the PP_i-dependent enzyme that may be involved in substrate binding.

Pyridoxal 5′-phosphate (PLP), which reacts specifically with lysine residues, can sometimes act as an affinity reagent for binding sites for phosphate esters as a result of the proximity of an aldehyde group with the phosphate of pyridoxal phosphate. Positively charged binding pockets that contain a lysyl residue can be identified following reduction of the Schiff base that is formed between the lysyl amino group and the aldehyde of pyridoxal phosphate. Cho and Cook (1988) reported that the PP_i-PFK from *Propionibacterium* was inactivated by pyridoxal phosphate and that the presence of any reactant decreased the rate of inactivation. These experiments were carried out without reduction so that the complex dissociated upon dilution. These results suggested the presence of lysine residues within the active site of PP_i-PFK that may be identified by reduction of the PLP–enzyme complex. In the current study, we have identified a single lysine residue whose modification by PLP brings about the loss of enzyme activity of the PP_i-dependent PFK. The position of this residue suggests an alignment of residues between PP_i-PFK and ATP-dependent PFK that differs from that suggested previously (Ladror et al., 1991).

Experimental Procedures

Enzymes and Substrates. Pyrophosphate-dependent phosphofructokinase (PP_i-PFK) from *Propionibacterium freudenreichii* was expressed in *E. coli* bearing the pLG1 phagemid (Ladror et al., 1991). Cultures were grown in LB containing 0.5 mM IPTG, and the cells were harvested by centrifugation. The enzyme was isolated and purified by methods previously described by Ladror et al. (1991).

Phosphofructokinase Assays. Enzymic activity for both native and modified PP_i-PFK was assayed at 30 °C and at pH 7.5 in a Gilford Response spectrophotometer. The assay medium contained 50 mM potassium 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (KTes) buffer, 0.1 mM EDTA, 3 mM magnesium chloride, 1 mM sodium pyrophosphate, 1.5 mM Fru-6-P, 0.2 mM NADH, 0.6 unit of aldolase, and 0.3 unit each of triosephosphate isomerase and glycerophosphate dehydrogenase. Reactions were initiated by the addition of PP_i-PFK. All reagents and assay auxiliary enzymes were purchased from Sigma.

For the determination of K_m values for Fru-6-P of both native and modified PP_i-PFK, the aforementioned assay medium was used except the concentration of Fru-6-P was varied between 0.073 and 2.89 mM. For the determination of K_m values for $MgPP_i$ of both native and modified PP_i-PFK, the aforementioned assay medium was used except the concentration of sodium pyrophosphate was varied between 4.9 and 971 μM and the concentration of Fru-6-P was held constant at 2.25 mM.

Pyridoxal 5′-Phosphate Inactivation. PP_i-PFK was incubated for 5 min at 25 °C with varying concentrations of pyridoxal 5′-phosphate (PLP) ranging from 0.156 to 12.5 mM in a solution containing enzyme at concentrations of 0.5–1.0 mg/mL and 125 mM KTes buffer at pH 7.0. The reaction was terminated by the addition of a freshly prepared solution of 270 mM sodium borohydride ($NaBH_4$) to a final concentration of 27 mM. Protection experiments were carried out in the aforementioned mixture with the addition of 10 mM fructose 1,6-bisphosphate (Fru-1,6-P_2) or 625 μM sodium pyrophosphate/1.25 mM magnesium chloride ($MgPP_i$). Control enzyme refers to a solution containing enzyme in 125 mM KTes buffer at pH 7.0 to which has been added $NaBH_4$ to a concentration of 27 mM. Aliquots were diluted prior to assay (100–200-fold) into a solution of 50 mM KTes buffer at pH 7.5 containing 0.1 mM EDTA.

Extinction Coefficient Determination. Three independent methods were used to obtain an extinction coefficient for PP_i-PFK.

(*A*) *By Bradford.* A sample of PP_i-PFK in 50 mM KTes, pH 7.5, containing 0.1 mM EDTA was diluted with an equal volume of water and dialyzed against two changes of 20 mM sodium chloride. The optical density (OD) at 280 nm of the dialyzed sample was recorded using a Gilford Response spectrophotometer, which was compared with the concentration of enzyme determined by the method of Bradford (1976) using bovine serum albumin as a standard. The OD at 280 nm and the molar concentration of PP_i-PFK, calculated from a mass of 43 243 daltons (Ladror et al., 1991), were used to calculate a molar extinction coefficient (ϵ_{280}) of 49 800 M^{-1} cm^{-1} for PP_i-PFK.

(*B*) *Theoretical Calculation.* A theoretical calculation of the extinction coefficient was based upon the known number of tyrosine and tryptophan residues in PP_i-PFK (Ladror et al., 1991) using the method of Edelhoch (1967). Twelve tyrosine residues and six tryptophan residues with ϵ_{280} values of 1280 and 5690 M^{-1} cm^{-1}, respectively (Edelhoch, 1967), were used to calculate a molar extinction coefficient of 49 500 M^{-1} cm^{-1} for the enzyme.

(*C*) *By Amino Acid Analysis.* An extinction coefficient calculation based upon amino acid analysis was performed as follows. A portion of the aforementioned dialyzed sample of enzyme in 20 mM NaCl was diluted with an equal part (v/v) of a 0.1 mM solution of norleucine added as an internal standard. Aliquots were dried and hydrolyzed in the vapor phase with 6 M HCl and 1% phenol under a nitrogen atmosphere for 24 and 48 h at 115 °C. The hydrolyzed samples were derivatized with phenyl isothiocyanate and the resulting PTH-amino acids were analyzed using a Waters PicoTag system according to the manufacturer's protocol. The concentration of protein was calculated from the concentration of each amino acid and its respective molecular weight minus one water for the peptide bond. The value obtained was then corrected based upon the yield of the internal standard norleucine in each analyzed sample. No correction was made for amino acid destruction or resistance to hydrolysis. Using the value for the concentration of PP_i-PFK and the OD reading at 280 nm, an ϵ_{280} of 51 900 M^{-1} cm^{-1} was secured. An average molar extinction coefficient of 50 400 M^{-1} cm^{-1} was calculated using the three independently obtained values from each of the aforementioned procedures. This average value was used to calculate the concentration of PP_i-PFK for all modification studies described herein.

Spectrophotometric Quantitation. PP_i-PFK reacted with varying concentrations of PLP in the presence and absence of substrates as described above was dialyzed overnight against 50 mM KTes buffer at pH 7.5 containing 0.01 mM EDTA. The dialyzed samples were applied to separate 1.0 × 25 cm columns of Sephadex G-50 equilibrated with same buffer. Elution was carried out with 50 mM KTes buffer at pH 7.5 containing 0.01 mM EDTA, and six to eight 3-mL fractions were collected from each column. Fractions containing enzyme were concentrated by lyophilization, and spectra were recorded from 210 and 410 nm using a Gilford spectrophotometer. The ratio of moles of incorporated PLP per mole of PP_i-PFK protomer was calculated using the OD readings at 280 and

325 nm for enzyme and phosphopyridoxylated lysine, respectively, with an ϵ_{280} of 50400 M^{-1} cm^{-1} for enzyme and an ϵ_{325} of 4800 M^{-1} cm^{-1} for phosphopyridoxyllysine (Lundblad & Noyes, 1984).

Tryptic Digestion. Enzyme was precipitated by the addition of trichloroacetic acid, collected by centrifugation, washed with water, and taken up in a solution of 6 M guanidine hydrochloride and 0.1 M *N*-ethylmorpholine at pH 8.2. Dithiothreitol (1.0 M aqueous) was added to a final concentration of 2 mM. The enzyme samples were carboxymethylated by the addition of a neutralized solution of 1.0 M aqueous iodoacetic acid to a final concentration of 50 mM. Samples were incubated at room temperature for ca. 30 min and were thereafter exhaustively dialyzed against two changes of 50 mM ammonium bicarbonate with 2 mM dithiothreitol in the first dialyzate. Trypsin in 1 mM HCl was added to a ratio of 1:25 (w/w protease to enzyme), and the digestion solutions were incubated overnight at 37 °C. The mixtures were lyophilized and the resulting powders stored at −20 °C prior to use.

HPLC Mapping and Peptide Purification. Lyophilized powders of tryptic-digested enzyme were taken up in 2.0% aqueous trifluoroacetic acid and filtered through Millipore Ultrafree-MC centrifugation filters. Peptides were separated by an Applied Biosystems 130A reverse-phase HPLC equipped with a Vydac C-18 2.1 × 150 mm column. Elution was carried out with a linear water/acetonitrile gradient containing 0.06% trifluoroacetic acid (1.0% acetonitrile/min) at a flow rate of 0.15 mL/min. Optical densities were measured at 214 and at 325 nm for the detection of phosphopyridoxyllysine. Desired fractions were collected by hand. Further purification of peptides was performed by decreasing the water/acetonitrile gradients in the region where the peptide of interest was eluted. Peak integrations were calculated automatically by a Hewlett-Packard HP 3396A integrator.

Protein Sequencing. Automated sequencing of peptides was performed in an Applied Biosystems 470A gas-phase protein sequencer using the standard sequencing program and the reagents provided by the manufacturer. The phenylhydantoin derivatives of amino acids liberated after each degradation cycle were identified and quantified by an on-line Applied Biosystems 120A HPLC equipped with a PTH C-18 column, using a modification of the procedure described by Hunkapiller (1985).

Molecular Sizing. The molecular sizing of the protein was determined on a Pharmacia FPLC fitted with a Superose 12 column equilibrated with 100 mM KTes (pH 7.5) containing 1.0 mM EDTA. The column was developed with a flow rate of 0.4 mL/min with the same buffer. A calibration curve was constructed using as standards cytochrome *c* (12.4 kDa), carbonic anhydrase (28 kDa), bovine serum albumin (63 kDa), aldolase (160 kDa), and thyroglobulin (660 kDa).

Results and Discussion

Extent of Modification and Effect of Phosphopyridoxylation on Activity. Samples of PP_i-PFK were reacted with varying concentrations of PLP, desalted, and spectrophotometrically analyzed as described under Experimental Procedures. The moles of incorporated PLP in each sample was determined using optical density readings at 325 nm as described under Experimental Procedures. As shown by the data in Figure 1, the incorporation of about 1 mol of PLP was observed at very low concentrations of PLP, whereas up to 3 mol was observed at much higher concentrations of reagent. A loss of enzymic activity (>70%) roughly parallels the incorporation of about 1 mol of PLP, suggesting that phosphopyridoxylation of a single lysine residue, which may be

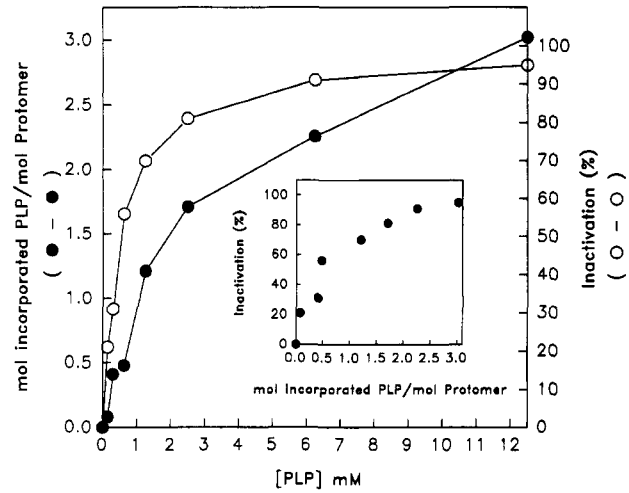

FIGURE 1: Moles of incorporated PLP per mole of PP_i-PFK protomer (●) and percent inactivation (○) as a function of PLP concentration. PP_i-PFK was treated with varying concentrations of PLP as described under Experimental Procedures. Samples were desalted, and optical densities (OD) were recorded from 210 to 410 nm. OD readings at 325 nm and an ϵ_{325} of 4800 M^{-1} cm^{-1} (Lunblad & Noyes, 1984) for a phosphopyridoxyllysine were used to calculate moles of incorporated PLP. OD readings at 280 nm and an ϵ_{280} of 50400 M^{-1} cm^{-1} for PP_i-PFK were used to calculate moles of PP_i-PFK protomer. Inactivation (percent) for each point was determined by assay as described under Experimental Procedures. Inset: Derivative plot of percent inactivation as a function of moles of incorporated PLP per mole of PP_i-PFK protomer.

Table I: Moles of Incorporated PLP per Mole of PP_i-PFK Protomer and Percent Inactivation for PP_i-PFK Modified with 1.25 and 12.5 mM PLP in the Absence and Presence of Substrates[a]

	[PLP] (mM)			
	1.25		12.5	
substrates	mol of PLP/mol of E[b]	inactn (%)[c]	mol of PLP/mol of E	inactn (%)
no additions	1.21	70	3.02	95
+10 mM Fru-1,6-P_2	0.71	24	1.88	73
+0.625 mM $MgPP_i$	1.02	63	3.98	93

[a] See Experimental Procedures for conditions and abbreviations. [b] Moles of incorporated PLP per mole of PP_i-PFK protomer. [c] Inactivation (%).

critical to enzymic activity, occurs more readily than PLP incorporation at other lysine residues. Modification of the enzyme with a high (12.5 mM) concentration of pyridoxal plus sodium borohydride resulted in the incorporation of about 0.5 mol of pyridoxal per mole of protomer (based on the extinction coefficient of pyridoxal phosphate) and the loss of only 10–15% of the initial activity. This suggests that the phosphate group may enhance the selectivity of the reagent because the incorporation of 0.5 mol of PLP resulted in the loss of more than 30% of the enzyme activity (see discussion of Figure 4 below).

Modification in the Presence of Substrates. To determine whether substrates can protect against modification and loss of activity, phosphopyridoxylation was carried out in the presence of substrates and products. Partial protection against activity loss was observed with Fru-6-P and Fru-1,6-P_2. Only minimal protection was seen with PP_i or $MgPP_i$. Fru-6-P plus magnesium methylene diphosphate provided no further protection than was seen with Fru-6-P alone. Table I provides results which show the extent of PLP incorporation (i.e., moles incorporation of PLP per moles of PP_i-PFK promoter) in the absence of and presence of 10 mM Fru-1,6-P_2 or 625 μM $MgPP_i$. The data were obtained from enzyme samples reacted with low (1.25 mM) and high (12.5 mM) concentrations of

PLP plus sodium borohydride in the absence and presence of substrates, assaying for activity, desalting, and spectrophotometric analysis for PLP content as described under Experimental Procedures. The addition of 10 mM Fru-1,6-P_2 prevents the incorporation of about 0.5 mol of PLP at low reagent concentration and about 1 mol of PLP at high reagent concentration in comparison with enzyme modified in the absence of substrate. Fru-1,6-P_2 partially protected against activity loss at low PLP concentration. At high PLP concentration, the presence of Fru-1,6-P_2 has a less dramatic effect, but there is nevertheless still some protection. On the other hand, $MgPP_i$ has no effect on PLP incorporation at low reagent concentration and a slight but reproducible protection against the loss of activity. There is no difference between the amount of inactivation at high concentrations of PLP in the absence and presence of $MgPP_i$. At high PLP concentration, more reagent is incorporated in the presence of $MgPP_i$ than in the absence of substrates. This could be due to a conformational change induced by $MgPP_i$ which might in turn expose additional lysine residues toward reaction with PLP.

Properties of Modified Enzyme. The observed inactivation caused by modification of PP_i-PFK by PLP plus $NaBH_4$ may be due either to modification of a lysine residue at the substrate binding site, which totally blocks the site and inactivates the enzyme, or to modification at some point or points distant from the active site that decreases the enzyme's affinity for substrate and leads to lower activity under the conditions of the assay. In order to differentiate between these two possibilities, a sample of PP_i-PFK modified with PLP at a concentration that produced about 80% loss of activity (see Figure 1) was chosen for further analysis. This modified enzyme was assayed at varying substrate concentrations to test for changes in affinity. Lineweaver–Burk plots of the relative rates of enzyme activity for phosphopyridoxylated PP_i-PFK and control PP_i-PFK obtained at varying concentrations of the substrate Fru-6-P with saturating concentrations of $MgPP_i$ exhibited two nonparallel lines which intercepted with the abscissa at two different values, but intercepted at the same point on the ordinate (results not shown). Similar plots were obtained using varying concentrations of $MgPP_i$ in the presence of saturating concentrations of Fru-6-P. The results showed that the activity loss caused by phosphopyridoxylation were due to a decrease in V_{max}. K_m values of 0.33 and 0.018 mM were secured for Fru-6-P and $MgPP_i$, respectively, for both phosphopyridoxylated PP_i-PFK and control PP_i-PFK. The data indicate that partial modification of a group of lysine residues in all of the enzyme molecules in any given sample of phosphopyridoxylated PP_i-PFK and a concurrent decrease in the ability of the enzyme to bind substrates do not account for the partial loss of activity. It is likely that the observed residual activity in a sample of PLP-modified enzyme results from PP_i-PFK that has not been phosphopyridoxylated at a lysine residue.

A possible mode of inactivation of PP_i-PFK would be the dissociation of the native dimeric form. To determine whether phosphopyridoxylation of PP_i-PFK causes the enzyme to dissociate into monomers, which in turn would result in the loss of enzyme activity, a sample of PP_i-PFK was modified with a high concentration of PLP (12.5 mM) and subjected to FPLC gel filtration under nondenaturing conditions as described under Experimental Procedures. The data (not shown) revealed that phosphopyridoxylated PP_i-PFK eluted with the retention time for a protein with a molecular mass of approximately 90 kDa, at a position identical to that seen with native PP_i-PFK. In addition, no peak was found which would correspond to a protein with a molecular mass of 43 kDa, which is the reported mass of the PP_i-PFK monomer (Ladror et al., 1991). The results indicate that PLP modification does not cause dissociation of the enzyme dimer.

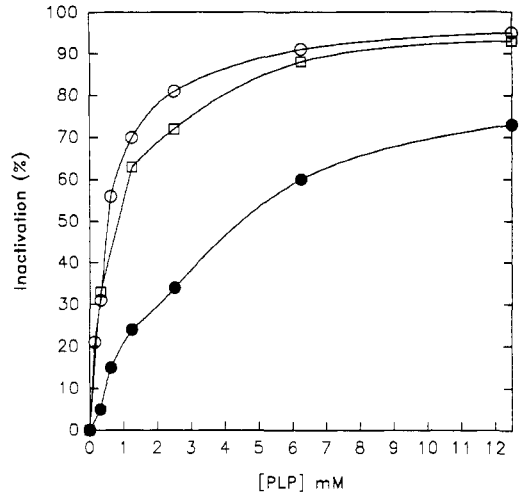

FIGURE 2: Inactivation (percent) of PP_i-PFK as a function of PLP concentration in the absence of substrate (○) and in the presence of 10 mM Fru-1,6-P_2 (●) or 625 μM $MgPP_i$ (□). Reaction conditions and the determination of inactivation (percent) by assay are described under Experimental Procedures.

Effect of the PLP Concentration on Activity and Protection by Substrate. A more complete description of the concentration dependence of inactivation in the absence and presence of substrates is shown in Figure 2. Each point on the curve represents a sample of enzyme which was reacted with PLP plus sodium borohydride in the absence and presence of 10 mM Fru-1,6-P_2 or 625 μM $MgPP_i$ as described under Experimental Procedures. The plot also clearly shows protection against inactivation was provided by Fru-1,6-P_2, especially at low concentrations of PLP, and that minimal protection was provided by $MgPP_i$. The results suggest that PLP readily modified at least one lysine residue located in the Fru-6-P binding site and that the presence of sugar phosphate partially shielded the residue against PLP modification.

HPLC Profiles of Tryptic Digests of PP_i-PFK Phosphopyridoxylated in the Absence and Presence of Substrates. To determine whether a single residue was indeed readily modified as opposed to the partial modification of a group of lysyl residues, it was necessary to identify all of those residues that were modified with and without the addition of protected ligands. As indicated by the data of Figure 2, the greatest relative protection by sugar phosphate was seen at low concentrations of PLP. For these reasons, the enzyme was modified with 1.25 mM PLP plus $NaBH_4$ in the absence and presence of 10 mM Fru-1,6-P_2 or 7.5 mM $MgPP_i$. Each sample was carboxymethylated, digested with trypsin, and resolved on reverse-phase HPLC as described under Experimental Procedures. The elution was monitored at an absorption of 325 nm, and the resulting profiles are shown in Figure 3. Profile 3A is for PP_i-PFK phosphopyridoxylated in the absence of substrates and displays a single major peak (asterisk) with only a small number of minor peaks. Some of these minor peaks also appear in profile D, which are the results of a control tryptic digest of unmodified PP_i–PFK and are therefore not attributed to any phosphopyridoxylated species. These data suggest that indeed under conditions where a significant loss of activity was observed (about 70% as indicated by Figure 2), most of the modification occurred at a single peptide and, one would presume, a single lysyl residue. Furthermore, as shown by profile C, this peptide was for the

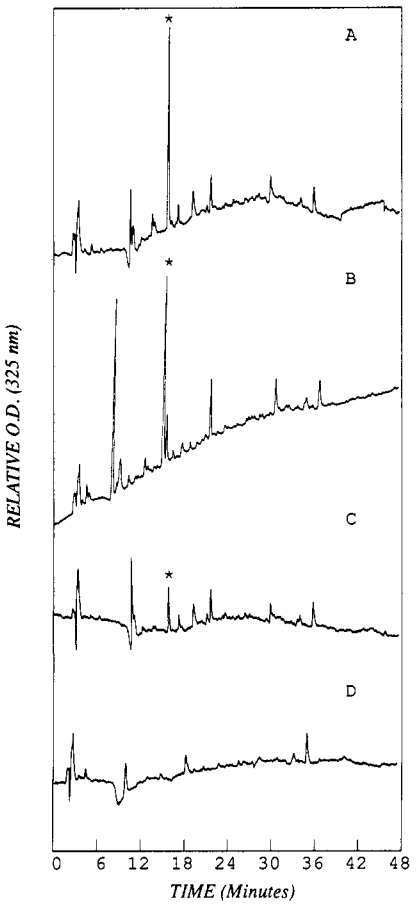

FIGURE 3: Reverse-phase HPLC profiles of tryptic digests of PP_i-PFK with optical densities recorded at 325 nm for the detection of phosphopyridoxyllysines. (A) PP_i-PFK reacted with 1.25 mM PLP; (B) PP_i-PFK reacted with 1.25 mM PLP in the presence of 7.5 mM $MgPP_i$; (C) PP_i-PFK reacted with 1.25 mM PLP in the presence of 10 mM Fru-1,6-P_2; (D) control enzyme as defined under Experimental Procedures. Protein samples were carboxymethylated, digested with trypsin, and resolved by reverse-phase HPLC as described under Experimental Procedures. The asterisk indicates the major peak of interest as addressed under Results and Discussion. In several apparently random samples, for example, profile B, a prominent peak eluted at approximately 11 min. This peak was further fractionated, and each fraction was subjected to amino acid analysis. It was concluded that this fraction contained no significant amount of peptide and was probably a reagent artifact.

most part protected by the presence of Fru-1,6-P_2. The eluted fraction corresponding to the major peak was collected, and the peptide with an absorbance at 325 nm was isolated by additional reverse-phase HPLC purification and sequenced to give IGAGXTMVQK, where X represents a modified residue. That the X corresponds to PLP-modified Lys-315 was confirmed by comparison of the sequence of this peptide with the published amino acid sequence for PP_i-PFK (Ladror et al., 1991). Coincident with the appearance of a major pyridoxylated peak at about 16 min was the disappearance of peptide that eluted at about 12 min as detected at 214 nm (not shown). This peptide was isolated from an unmodified preparation of enzyme, purified, and subjected to gas-phase sequence analysis to provide the sequence TMVQK. This represents one of the two precursor peptides to the major pyridoxylated peptide and thus would be expected to disappear following pyridoxylation.

An additional important feature of Figure 3 is the presence of the major peak (asterisk) at approximately 16 min in profile B for PP_i-PFK phosphopyridoxylated in the presence of $MgPP_i$. The peak has a relative OD ca. equal to that for the major peak of profile A.

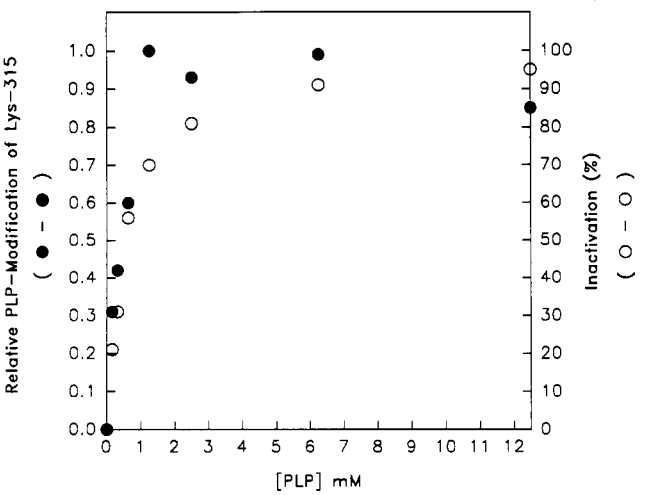

FIGURE 4: Relative extent of PLP modification of Lys-315 (●) and percent inactivation (O) as a function of PLP concentration. Samples were modified with varying concentrations of PLP, desalted, digested with trypsin, and resolved on HPLC as described under Experimental Procedures. Data were normalized by comparing the OD at 325 nm for the peptide containing Lys-315 with the combined OD at 214 nm of three other well-resolved peaks as described under Results and Discussion. Percent inactivation for each point was determined by assay as described under Experimental Procedures.

Extent of Phosphopyridoxylation of Lys-315. If the loss of enzyme activity was the result of modification of Lys-315, then a direct correlation should be observed between the extent of modification and the loss of activity. To test this hypothesis, tryptic digests of enzyme samples phosphopyridoxylated in the presence of varying concentrations of PLP, which were used to generate Figure 1, were resolved on reverse-phase HPLC (data not shown). Optical densities were measured at 214 and 325 nm. Peaks on the HPLC profiles were integrated as described under Experimental Procedures. The data which signify the extent of PLP modification of Lys-315 were obtained as follows. The residue was assumed to be fully modified at high concentrations of PLP. The validity of this assumption was based on the fact that an unmodified precursor to the modified peptide, the peptide that eluted at about 12 min in HPLC profile, could be monitored at 214 nm. The virtual complete disappearance of this peak following modification of unprotected enzyme at PLP concentrations of 6 mM or above indicated the near-total modification of Lys-315. Under the conditions of modification at high concentrations of PLP, the area under the peak bearing Lys-315 could be set at 100. To normalize the areas for all of the samples, the integration value for the peak at 325 nm corresponding to the peptide which contains Lys-315 was divided by the combined integration values for three other well-resolved peaks from the HPLC profile for the same sample obtained at 214 nm. The peaks from the HPLC profiles obtained at 214 nm were chosen on the basis of the observation that increasing concentrations of PLP had no effect on the ratios of these peaks to each other, indicating that the relative amounts of the corresponding tryptic peptides did not change. Therefore, the tryptic peptides used for comparison were constant in comparison with the increasing amount of peptide containing Lys-315. The amount of phosphopyridoxylated Lys-315 increased rapidly with increasing PLP concentration as evinced in Figure 4. This increase in modified Lys-315 was paralleled by a rapid increase in the amount of inactivation. These results strongly imply that Lys-315 is critical to the activity of PP_i-PFK. Although other lysine residues in PP_i-PFK were modified with PLP, none was modified as extensively as Lys-315 nor did their modification closely parallel activity loss.

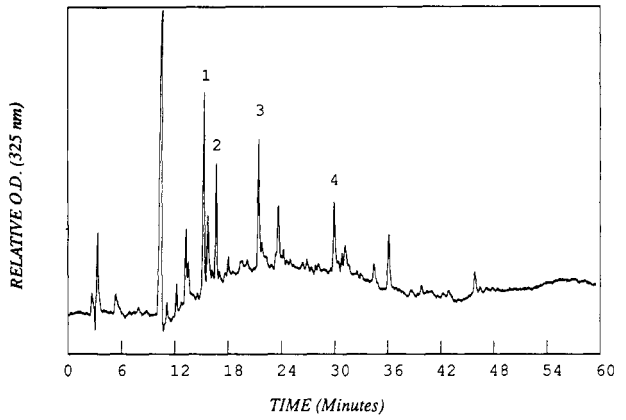

FIGURE 5: HPLC profile of a tryptic digest of PP_i-PFK modified with a high concentration (12.5 mM) of PLP as described under Experimental Procedures. PLP-modified protein was desalted, carboxymethylated, digested with trypsin, and resolved on HPLC as described under Experimental Procedures. Optical densities were monitored at 325 nm for the detection of peptides containing phosphopyridoxyllysines. Numbers correspond to peptide fractions which were further purified and subjected to amino acid sequencing. Peptide sequences are given under Results and Discussion. Unnumbered peaks either may be accounted for in the HPLC profile of control enzyme (see Figure 3) or are too small to be of any significance.

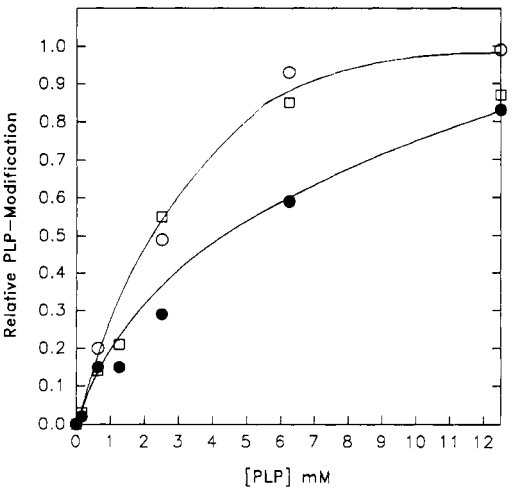

FIGURE 6: Relative extent of PLP modification of Lys-80 plus Lys-85 as a function of PLP concentration in the absence (○) of substrates and in the presence of 10 mM Fru-1,6-P_2 (□) or 625 μM $MgPP_i$ (●). Samples were treated as described in Figure 4, with the addition of the substrates Fru-1,6-P_2 or $MgPP_i$. Data were normalized by comparing the combined OD at 325 nm for the peptides containing Lys-80 and -85 with the combined OD at 214 nm of three well-resolved peaks as described under Results and Discussion.

Identification of Other Reactive Lysine Residues. Modification of PP_i-PFK in the presence of low concentrations of PLP (≤1.25 mM) occurs predominantly at Lys-315 with only scattered minor phosphopyridoxylation occurring at other lysine residues (see Figure 3). These minor sites of modification were reproducible from sample and thus represent residues that are modified more readily than the other 12 lysyl residues of PP_i-PFK and may indicate particularly exposed regions of the enzyme. To identify these additional sites that are reactive with PLP, the modification reaction at a high concentration of PLP (12.5 mM) was studied more extensively. In Figure 5 is shown the reverse-phase HPLC profile monitored at 325 nm for a tryptic digest of a sample of PP_i-PFK modified with a high concentration of PLP. Peak 1 corresponds to the peptide which contains phosphopyridoxylated Lys-315. Peaks 2–4 correspond to peptides that contain additional lysine residues which appear to have been modified less extensively in comparison with Lys-315 but were present in all reaction mixtures, with and without the addition of protective ligands. The sequences of the peptides which contain these additional modified lysine residues (indicated as X) are VXLTNVK for peak 2, where the modified residue is Lys-80, LTNVXDLVAR, containing Lys-85, and INPGAWFAXQFAER for peak 4 where X is Lys-305. The phosphopyridoxylated lysine residue (X) was indicated by the absence of a known PTH-amino acid recovered at this cycle during the sequence analysis and by the known presence of lysine at this position in the native enzyme (Ladror et al., 1991).

Modification of Lys-80 and -85. It should be noted that Lys-80 and Lys-85 represent consecutive basic residues in the enzyme sequence. Because no peptide was found that had both lysyl residues modified, the modification of these residues would appear to be mutually exclusive. The sum of the integration values for the corresponding HPLC peaks obtained at an OD of 325 with a sample of PP_i-PFK modified in the presence of high PLP concentrations were approximately equal to the integration value of the peak for the peptide which contains Lys-315, suggesting that at high concentrations of PLP a combined total of 1 mol of PLP reacted between each of the two lysine residues. This was confirmed by the virtual disappearance of a peptide that eluted at about 13 min in the HPLC profile. Analysis of this peptide in an unmodified sample indicated that it was LTNVK, a peptide that would be lost upon the modification of either Lys-80 or Lys-85. Integration data for the sum of the peak areas corresponding to peptides containing Lys-80 and -85 from HPLC profiles obtained at 325 nm of tryptic digests of PP_i-PFK modified with varying concentrations of PLP in the absence and presence of Fru-1,6-P_2 or $MgPP_i$ were normalized as outlined in a previous section. The plot of moles of modified Lys-80 plus -85 against varying concentrations of PLP (Figure 6) indicated that $MgPP_i$ provided some protection against phosphopyridoxylation of these lysine residues. The results summarized in Table I show that the presence of $MgPP_i$ also provided slight protection against PLP inactivation especially at low concentrations of the reagent, an observation that was observed repeatedly. This protection by $MgPP_i$ against activity loss may have resulted from the protection against PLP incorporation at Lys-80 and -85.

Sequence Alignment Based upon Modification of Lys-315. In a previous alignment of PP_i-PFK with allosteric ATP-dependent PFK from *E. coli*, three basic residues involved in the binding of the 6-PO_4 group of a sugar phosphate substrate could not be aligned (Ladror et al., 1991). These three residues were Arg-243, His-249, and Arg-252. A His residue at position 291 in PP_i-PFK was aligned with His-249 of the enzyme from *E. coli*, but the alignment of these residues was reported with little confidence. An alternative sequence alignment based upon results presented in the current study is shown in Figure 7. The alignment of the entire amino halves of the molecules remains the same as that which was reported by Ladror et al. (1991). Changes were only made in the carboxyl halves with Lys-315 of PP_i-PFK having been specifically aligned with Arg-243 of PFK from *E. coli*, a residue known to be involved in the binding of 6-PO_4 of Fru-6-P to PFK from *E. coli* (Shirakihara & Evans, 1988). Substitution of a lysine for an arginine is a conservative change in that both are basic residues and capable of being cationic in their respective conjugate acid forms. Evidence presented herein strongly suggests that Lys-315 is located in the sugar phosphate binding site of PP_i-PFK. It is therefore concluded that Lys-315 of PP_i-PFK functions in the same manner as

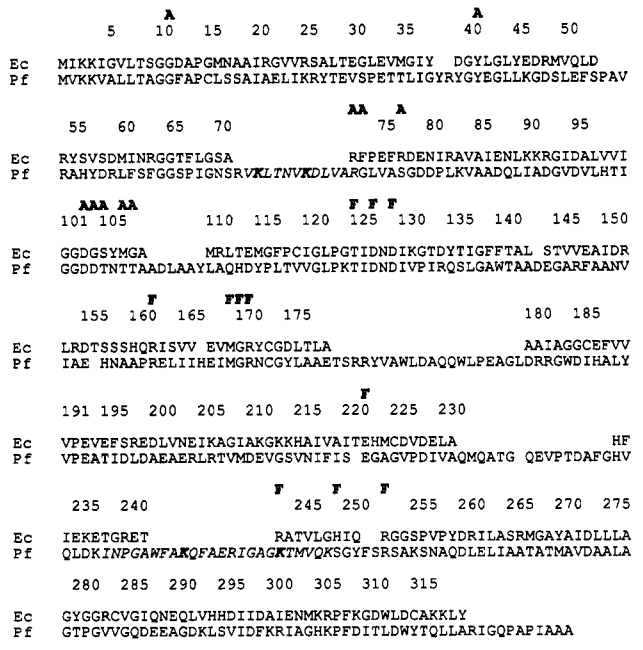

FIGURE 7: Proposed sequence alignment for allosteric ATP-dependent *E. coli* PFK and PP_i-PFK from *Propionibacterium* based upon modification of Lys-315. Amino acids are indicated by the single-letter code. Spaces indicate gaps, and boldface characters represent residues modified by PLP. Characters F and A above the sequences show the residues that contribute to the Fru-6-P and ATP binding sites of *E. coli*, respectively. The *E. coli* sequence is from Shirakihara and Evans (1988), and the *Propionibacterium* sequence is from Ladror et al. (1991). Abbreviations: Ec, *E. coli*; Pf, *P. freudenreichii*.

Arg-243 in *E. coli* PFK, that is, to bind the 6-PO_4 of sugar phosphate substrates, hence the specific alignment of these two residues. In support of the contention that the residue is implicated in the binding of the 6-PO_4 is the observation that both fructose-6-P and the bisphosphate protect against modification. As a consequence of this alignment, Arg-326 of PP_i-PFK has been aligned with Arg-252 of *E. coli* PFK. The new alignment was performed by eye and has the same number of gaps as that proposed by Ladror et al. (1991). In addition, the overall percent identity between the molecules does not significantly change from that which was previously reported (Ladror et al., 1991). Whereas the new alignment does not significantly change the overall percent identity between the molecules, it does provide for an alignment of basic residues which may be involved in the binding of the 6-PO_4 group of sugar phosphate substrates. The majority of phosphopyridoxylation of PP_i-PFK seems to be confined to two regions of the molecule as represented by the italicized characters in the PP_i-PFK sequence in Figure 7.

Although four out of seven residues which form the ATP binding site of all known ATP-dependent PFKs are conserved in PP_i-PFK (and are found in the amino halves of the molecules), the enzyme does not utilize the nucleotide as a phosphoryl donor. One of these four conserved residues, Arg-72 corresponding to Arg-90 of PP_i-PFK, interacts with one of the phosphates of ATP and may well interact with PP_i. It should be noted that this residue is immediately adjacent to the lysine-containing segment that was modified by PLP. While the protection data for Lys-80 and -85 are clearly not as convincing as they are for Lys-315, the partial protection of these residues by PP_i coupled with the possible homology with ATP-dependent PFK suggests that these lysine residues may indeed lie at or near the PP_i binding site.

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