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AFFINITY LABELING OF THE COFACTOR SITE IN GLYCOGEN PHOSPHORYLASE b WITH A PYRIDOXAL 5'-PHOSPHATE ANALOG

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SUMMARY

P^1,P^2-bis(5'-pyridoxal)diphosphate inactivates apophosphorylase b from rabbit muscle, but not holophosphorylase. Inactivation is stoichiometric with the incorporation of 1 mol of the pyridoxal 5'-phosphate analog per mol of enzyme monomer. One of the two pyridoxal groups of the analog is linked to the cofactor site forming a Schiff base, and is not reduced with $NaBH_4$. The other also forms a Schiff base, but is easily reduced by the same treatment. The residue involving in the latter binding has been identified as Lys-573. Its ε-amino group may interact with the phosphate group of the cofactor or of the substrate in the native enzyme.

Glycogen phosphorylase (EC 2.4.1.1) contains pyridoxal 5'-phosphate (PLP)[1] which is required for activity. Although a conformational role of this cofactor was clearly demonstrated (1), attempts to reveal its direct involvement in catalysis have all failed. However, recent experimental results (2-4) enable us to discuss the catalytic function of this cofactor, especially of its phosphate group. Furthermore, the cofactor has recently positioned both in the amino acid sequence (5) and in the three-dimensional structure (4); it is bound to Lys-679 and buried inside the protomer adjacent to the substrate site. However, the detailed characterization of the sites is lacking. On the basis of our recent finding that the cofactor site is in a relatively large hydrophobic region (6), we have designed PLP derivatives with chromophores at the 5'-phosphate to explore the microenvironment of the cofactor and substrate sites in phosphorylase.

[1]Abbreviations : PLP, pyridoxal 5'-phosphate; bis-PLP, P^1,P^2-bis(5'-pyridoxal) diphosphate.

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In the present study we synthesize and use bis-PLP, a PLP analog linking two PLP groups through a pyrophosphate linkage (Fig. 1), to test its ability to label the cofactor site in glycogen phosphorylase from rabbit muscle.

MATERIALS AND METHODS

Enzymes. Rabbit muscle phosphorylase b was prepared according to Fischer and Krebs (7). The apoenzyme was obtained by the procedure of Shaltiel et al. (8). Phosphorylase activity was measured in the direction of glycogen synthesis with the reaction mixture containing 50 mM glucose 1-phosphate, 1 % glycogen, 1 mM AMP, 25 mM sodium glycerophosphate and 25 mM mercaptoethanol, at 25°C and pH 7.0.

Synthesis of bis-PLP. Bis-PLP was synthesized by the anion-exchange method of Michelson (9). He showed that P^1-nucleoside-5' P^2-diphenyl pyrophosphate synthesized from nucleoside 5'-phosphate and diphenyl phosphochloridate is attacked by an anion in the presence of pyridine, the diphenyl phosphate group being displaced by the anion. We synthesized P^1-pyridoxal-5' P^2-diphenyl pyrophosphate from triethylammonium PLP and diphenyl phosphochloridate, and obtained bis-PLP through displacement of the diphenyl phosphate group by the phosphate group of PLP. R_f values of PLP and bis-PLP on TLC (Kieselgel, Merck) were 0.35 and 0.48, respectively, with the solvent (n-butanol:pyridine:water = 6:4:3). A more detailed method of the synthesis will be published elsewhere (6).

Assay of pyridoxal contents in protein. Pyridoxal contents were assayed essentially according to the phenylhydrazine method (10). A sample solution (1 ml) was mixed with 6 M guanidine·HCl (1 ml), phenylhydrazine (0.2 g/40 ml 5 N H_2SO_4; 0.4 ml) and conc. H_2SO_4 (0.4 ml), and heated at 90°C for 30 min. After filtration, the absorbance at 417 nm was measured. In the assay of the pyridoxal contents of deproteinized samples, 5 % trichloroacetic acid (1.5 ml) was added to a sample solution, and stood for 30 min at 0°C. After centrifugation, the supernatant was used for the assay as described above.

Preparation of bis-PLP-labeled phosphorylase. Apophosphorylase (66 μM) and bis-PLP (67 μM) were mixed in 0.1 M sodium glycerophosphate-20 mM mercaptoethanol buffer (40 ml) at pH 7.0. After standing overnight, 0.1 M $NaBH_4$ (0.4 ml) was added with mild stirring. After 30 min another 0.4 ml of 0.1 M $NaBH_4$ was added and stood for 4 hr. Then solid guanidine·HCl and EDTA were added to the protein solution to final concentrations of 6 M and 1 mM, respectively. The solution (80 ml) was incubated at 35°C for 30 min, and then 1 M NH_2OH (1 ml) was added. After further 30 min 0.2 M $NaBH_4$ (1 ml) was added. The solution was concentrated to a small volume and dialyzed against 6 M guanidine·HCl. Through this procedure, one of the two pyridoxal groups was covalently fixed to protein, and the other converted to a pyridoxamine derivative.

Identification of the labeled site with bis-PLP. Peptide purification, amino acid analyses, Edman degradations, and carboxypeptidase A digestions were performed as described previously (11,12). The bis-PLP-labeled phosphorylase (2 μmol) was carboxymethylated by the procedure of Crestfield et al. (13). The product was suspended in 0.15 M NH_4HCO_3 (pH 8.5), and digested with 4 mg of α-chymotrypsin (Worthington) at room temperature for 6 hr with stirring. The labeled peptide was purified by a series of Bio-Gel P-4 column chromatography, paper electrophoresis at pH 6.5, and paper chromatography twice with n-butanol: pyridine:acetic acid:water = 15:10:3:12 and pyridine:isoamyl alcohol:0.1 N NH_4OH = 6:3:5. The label was detected by the Pauly reaction (12).

RESULTS AND DISCUSSION

Bis-PLP contains two reactive aldehyde groups, and can react with two

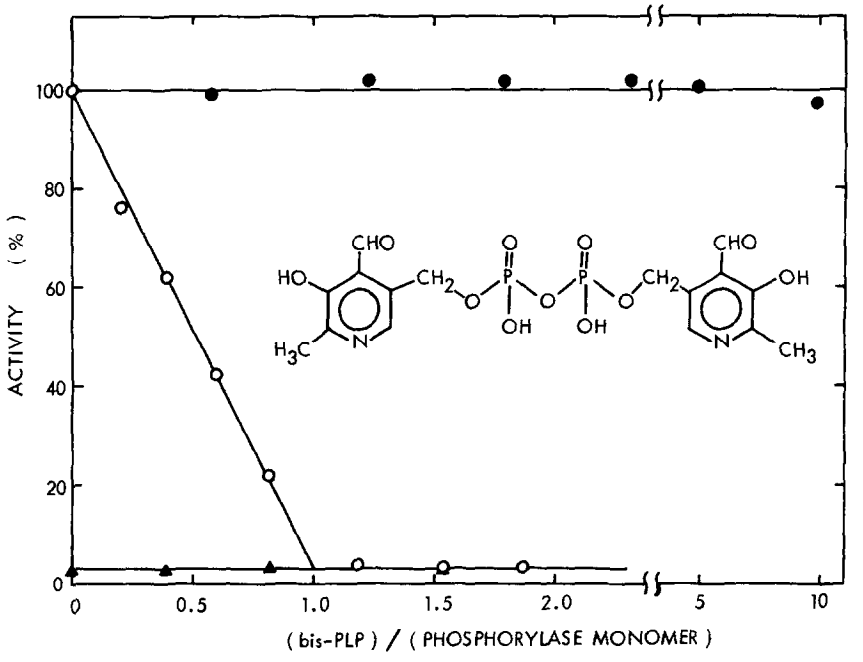

Figure 1 : Effect of bis-PLP on the activity of phosphorylase b. Activity of the apoenzyme (3.8 μM) after incubation with bis-PLP for 1 hr at 25°C in 0.1 M glycerophosphate-20 mM mercaptoethanol at pH 7.0 (▲); activity of the bis-PLP-bound apoenzyme treated with a 5-molar excess of PLP for 30 min (○); activity of the holoenzyme treated with bis-PLP for 1.5 hr (●).
Inset: Structural formula of bis-PLP.

lysine residues forming a cross-linked Schiff base derivative. Figure 1 shows the effect of the compound on the activity of phosphorylase b. It neither inactivates the holoenzyme nor activates the apoenzyme. However, preincubation of the apoenzyme with bis-PLP for 1 hr resulted in a loss of activity depending on the amount of the compound, while the activity of the intact apoenzyme was fully recovered after incubation with PLP for 30 min. No further inactivation of bis-PLP-bound apoenzyme was induced even after 21 hr. The reconstitution of the apoenzyme with PLP was completely blocked by incubation with an equimolar amount of bis-PLP to enzyme monomer. In incubation of the bis-PLP-bound apoenzyme with an excess of PLP for 21 hr, no recovery of activity was observed.

Bis-PLP bound to apophosphorylase showed two main absorption peaks at 335 and 415 nm (Fig. 2). The latter band is characteristic to the Schiff base. The circular dichroism spectrum above 250 nm of the bis-PLP-bound apoenzyme

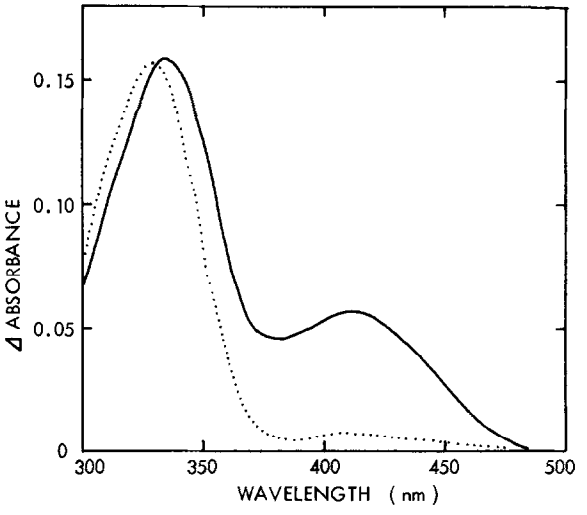

Figure 2 : Absorption spectra of bis-PLP-bound apophosphorylase. Difference absorption spectrum of the apoenzyme (24.8 μM) in the presence of 20.5 μM bis-PLP versus the apoenzyme (———); difference spectrum of the bis-PLP-bound apoenzyme treated with 2.3 mM $NaBH_4$ versus the apoenzyme (·····).

greatly differed from that of the apoenzyme, and resembled that of the holoenzyme (14). The results of the spectrometric titrations clearly indicate the formation of an equimolar complex of bis-PLP with apophosphorylase (Fig. 3).

On the $NaBH_4$-treatment of the bis-PLP-bound apophosphorylase, absorption at 415 nm decreased to 10 % of the original, and no further decrease was observed on the additional treatments (Fig. 2). However, the same treatment resulted only a 51-52 % loss of the pyridoxal group of bis-PLP. On the denaturation of the $NaBH_4$-treated bis-PLP-apoenzyme with trichloroacetic acid, 70-80 % of the pyridoxal groups remained in protein, indicating that almost all the bis-PLP molecules were covalently fixed to protein with reduction. PLP in native phosphorylase strongly absorbs at 335 nm and weakly at 415 nm (14,15), and is not reduced by $NaBH_4$ under the mild conditions. These results indicate that bis-PLP crosslinks two different lysine residues in an enzyme monomer, forming two Schiff base structures with different characteristics. One of the two pyridoxal groups mainly absorbs at 335 nm and is resistant against $NaBH_4$-reduction, like PLP in native phosphorylase. The other showing a different absorption spectrum is subject to the reduction, through which a lysine residue

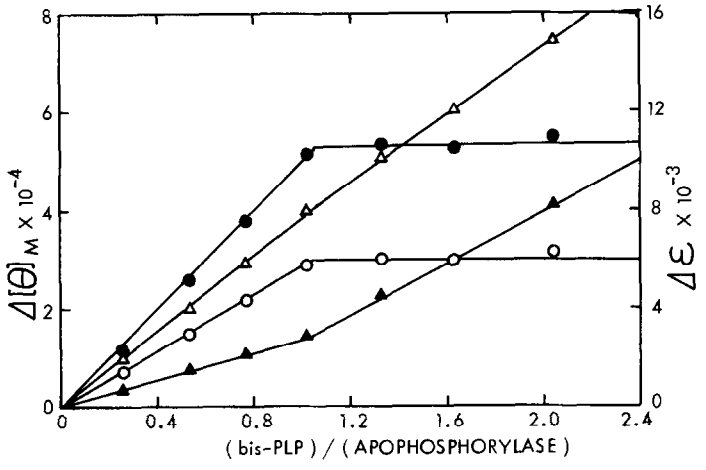

Figure 3 : Titration of apophosphorylase with bis-PLP. The enzyme concentration was 10.6 μM. Molar ellipticity change at 259 (●) and 335 nm (○). Molar absorbance change at 335 (△) and 415 nm (▲).

in protein can be labeled.

Location of the site labeled with bis-PLP after the mild $NaBH_4$-treatment was determined using the bis-PLP-labeled phosphorylase prepared as described in MATERIALS AND METHODS. Figure 4 shows the elution pattern of gel chromatography of the chymotryptic digest of the bis-PLP-labeled enzyme. The peptide containing the label was eluted as a single peak. Several fluorescent spots under an ultraviolet lamp were obtained on the following purification steps. However, only a single spot among them on each purification step was Pauly-positive (yellow) on paper. Other fluorescent spots yielded after acid hydrolysis neither pyridoxyllysine nor other amino acids. Thus, the bis-PLP derivative is thought to be fairly unstable and gradually degraded during purification. Its lability reasonably explains a low yield (4 %) of the labeled peptide. The amino acid composition of the labeled peptide were Lys(Pxy) 0.56, Arg 0.93, Glu 1.21, Leu 1.86. The first step of Edman degradation gave a Pauly-positive spot (reddish orange) on a TLC plate, remained at the origin after development with the solvent **V**, like the PTH derivative of pyridoxyllysine (12) but unlike PTH-histidine or PTH-tyrosine. It is probably the PTH derivative of labeled lysine. The second and third steps gave PTH-arginine and PTH-glutamine, respec-

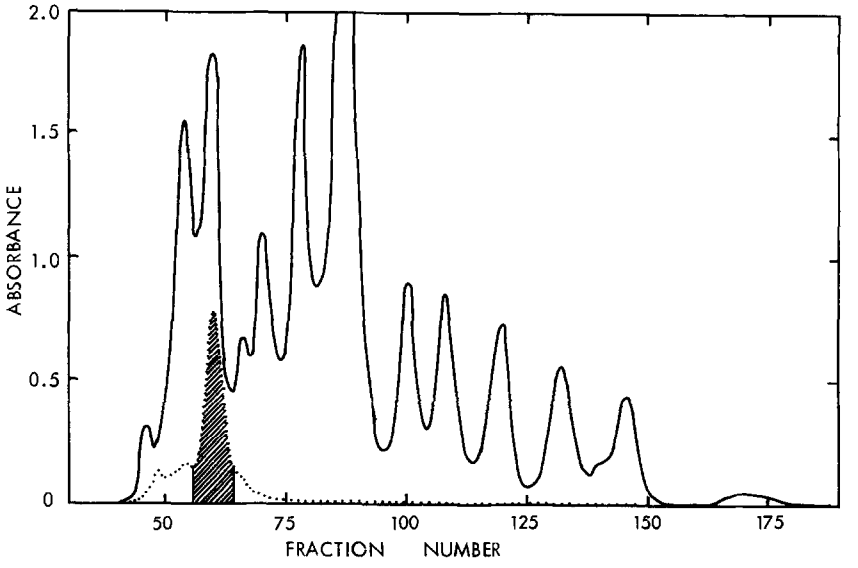

Figure 4 : Elution pattern of the chymotryptic digest of bis-PLP-labeled phosphorylase on an Bio-Gel P-4 column (2 X 180 cm) equilibrated with 0.2 M NH_4HCO_3-NH_4OH at pH 9.0. The flow rate was 8.5 ml/hr. Fractions of each 2.8 ml were monitored by absorption at 280 (———) and 310 nm (·····). The hatched areas shows the fractions collected for further purification.

tively. Carboxypeptidase A digestion yielded only leucine. Thus, the sequence is Lys(labeled)-Arg-Gln-Leu-Leu. This coincides only with the published amino acid sequence from Lys-573 to Leu-577 (Ref. 5). Tyr-572, the amino terminal side of Lys-573, satisfies the specificity of α-chymotrypsin. Thus, Lys-573 is the labeled site with bis-PLP in phosphorylase.

The PLP derivatives with bulky substituents at the 5'-phosphate, such as pyridoxal 5'-phosphate monobenzyl ester and pyridoxal 5'-diphosphate β-monophenyl ester, bound to the cofactor site in phosphorylase in the same binding mode as the natural cofactor PLP (6). Bis-PLP reacted with the apoenzyme, but not the holoenzyme, and it retarded the reconstitution of the apoenzyme with PLP. One of the pyridoxal groups of the bis-PLP bound to apophosphorylase showed the same resistance against $NaBH_4$-reduction and similar absorption and circular dichroism spectra to the PLP in native phosphorylase. It is concluded that bis-PLP crosslinks Lys-679, the original cofactor site (5), and Lys-573 in phosphorylase. Thus, Lys-573 is located adjacent to the cofactor site, and its ε-amino group may interact with the phosphate group of the cofactor or of the

substrate in native phosphorylase.

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