Function of the Phosphate Group of Pyridoxal 5'-Phosphate in the Glycogen Phosphorylase Reaction*

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To understand the catalytic mechanism of glycogen phosphorylase (EC 2.4.1.1), pyridoxal(5')phospho(1)-β-D-glucose was synthesized and examined as a hypothetical intermediate in the catalysis. Pyridoxal phosphoglucose bound stoichiometrically to the cofactor site of rabbit muscle phosphorylase b in a similar mode of binding to the natural cofactor, pyridoxal 5'-phosphate. The rate of binding of pyridoxal phosphoglucose was only 1/100 compared with that of pyridoxal phosphate. The enzyme reconstituted with pyridoxal phosphate showed no enzymatic activity at all even after prolonged incubation of the enzyme with substrates and activator. The present data would contradict participation of the phosphate group of pyridoxal phosphate in a covalent glucosyl-enzyme intermediate even if the covalent intermediate was formed during the catalysis.

Phosphorylase (EC 2.4.1.1) catalyzes the transfer of a glucosyl group between α-glucose-1-P and the nonreducing end of an α-1,4-linked glucosyl polymer, with retention of configuration. The observation of 10O exchange between the ester and phosphoryl oxygens of glucose-1-P suggests for the enzyme a double displacement mechanism involving the formation of a glucosyl-enzyme intermediate (1). The intermediate can be either a carbonium ion stabilized by a nucleophilic group on the protein or an enzyme-bound glucoside with β configuration.

The topology of the active site has been elucidated by both x-ray crystallographic (2, 3) and modification studies on the cofactor site (4); all three substrates are bound near the phosphate group of the cofactor, pyridoxal 5'-phosphate (PLP). Johnson et al. (6) have recently pointed out on the basis of model building experiments that the phosphate group of the cofactor is the most likely candidate for the nucleophilic group interacting with the glucosyl intermediate.

It is, therefore, of interest and essential to the understanding of the catalytic mechanism to examine formation of the glucosyl-enzyme intermediate bound through the phosphate group of the cofactor. In this paper, we have investigated this possibility by synthesizing chemically the proposed covalent intermediate, β anomic pyridoxal phosphoglucose (PLP-β-Glc), and replacing the enzyme-bound cofactor with this compound.

EXPERIMENTAL PROCEDURES

Reconstitution experiments of rabbit muscle apophosphorylase b with the PLP derivative were carried out at 35°C in 0.1 M sodium 2-glycerophosphate buffer (pH 7.0) containing 20 mM mercaptoethanol as previously described (6). The enzyme was assayed at 39°C in the direction of glycogen synthesis (7). The assay medium contained 2.9 to 7.3 μg/ml of phosphorylase, 75 mM glucose-1-P, 16 mg/ml of shellfish glycogen, 1 mM AMP, 0.5 mg/ml of bovine serum albumin, 20 mM mercaptoethanol, and 0.1 M sodium maleate buffer, pH 6.5. Thin layer chromatography on a Kieselgel 60 (Merck) was performed using two solvent systems: Solvent A, 1-butanol/formic acid/water (13:3:3); Solvent B, 1-butanol/pyridine/water (6:4:3). CD and H NMR spectra were recorded with a Jasco J-20 and a Jeol JNM-FX 100, respectively.

Synthesis of PLP-β-Glc—The König-Knorr reaction was applied to synthesize the β anomer of pyridoxal phosphoglucose since the reaction of a per-O-acylated α-glucosyl halide with a nucleophile in the presence of a heavy metal salt as acid acceptor proceeds via net inversion of configuration at the anomeric carbon producing the corresponding β anomeric glycoside (8). The synthesis was performed in the dark as far as possible. All column chromatographic steps were carried out at 4°C. Under anhydrous conditions, 18 g (44 mmol) of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (9) and Ag2CO3 (6 g) were mixed with PLP-H2O (3.45 g, 1 mmol) dissolved in CHCl3 (17 ml) and triethylamine (2 ml). The mixture was agitated in a rotary shaker at 30°C for 3 h. The precipitated salt was removed by centrifugation. The CHCl3 solution was extracted with water. Then the aqueous solution was applied to an Amberlite CG-50 (H+) column (2.6 x 23 cm). The tetracetylated pyridoxal phosphoglucose (Rf = 0.64 with Solvent A, 0.74 with B) was eluted with water after unreacted PLP. Appropriate fractions were combined, neutralized with triethylamine, and concentrated to a few milliliters. Then deacetylation was performed by addition of 5 ml of 10% NaOH at room temperature. After 5 min, 30 ml of cold water was added and the solution was neutralized with Amberlite CG-50 (H+)柱. After filtration, the solution was applied to a Dowex 50-X8 (H+) column (2.6 x 17 cm) and eluted with water. The chromatographic step was efficient to remove the impurities composed of sugar and pyridine ring but not to remove the acetic acid. Then fractions containing pyridoxal phosphoglucose (Rf = 0.32 with A, 0.61 with B) were combined, neutralized with triethylamine, evaporated to remove the water, redissolved in a small volume of ethanol, and extracted with ether to remove the triethylammonium acetate. The remaining residue was dissolved in water, applied to the second Dowex 50-X8 (H+) column (1.8 x 11 cm), and eluted with water. The eluate was neutralized with KOH, evaporated, and then redissolved in a small volume of ethanol. The yellow potassium salt (204 mg) of pyridoxal phosphoglucose was precipitated by addition of ether to the ethanol solution. The product was analyzed as a mixture of hydrate and ethyl hemiacetal:

C24 H22 N O6 K P
Calculated: C 66.22 H 5.07 N 2.62 P 6.23
Found: C 66.35 H 5.18 N 2.75 P 6.89

The UV spectra (nm) showed λmax 293 (ε 6900) and 337 (ε 1200) in 0.1 M HCl, λmax 335 (ε 2200) and λmax 388 (ε 5600) in 0.1 M sodium phosphate buffer at pH 7.0, and λmax 310 (ε 850) and 388 (ε 6400) in 0.1 M NaOH. In functional group analyses, 1.03 mol of pyridoxal and 1.00 mol of...
glucose/mol of pyridoxal phosphoglucose were detected by the phenylhydrazine-sulfuric acid method (the hydrazone reaction was performed at 90°C for 30 min) (10) and by the phenol-sulfuric acid method, respectively. [α]_D^20 = −7.3° (c, 0.49, water) (cf. the value (11) of glucose-1-P in water is +78°). The NMR spectrum (in D_2O, internal standard sodium 3-(trimethylsilyl)-1-propanesulfonate, δ in ppm) showed 10.4, 1 H singlet of 4-CHO; 7.70, 1 H singlet of 6-H; 5.61, 2 H doublet of 1'-H of glucosyl group (J_HH = J_HH = 7.3 Hz); 2.46, 3 H singlet of 2-CH_3; 1.17, 3/2 H triplet of CH_3 of ethanol (J = 7.3 Hz), as shown in Fig. 1 (cf. the NMR spectrum (12) of glucose-1-P in D_2O shows 5.4, quartet (double doublets) of 1-H (J_HH = 3.0 Hz, J_HH = 7.0 Hz). The value of the spin-spin coupling constant of 1'-H and 2'-H of pyridoxal phosphoglucose is larger than the corresponding value of glucose-1-P. This suggests that the glucosyl moiety of pyridoxal phosphoglucose is in a C1 conformation and that the PLP derivative is a β anomer. Since the proton of an axially oriented hydrogen is known, as the empirical rule, to come into resonance at a higher field than that of an equatorially oriented hydrogen (13), the observed chemical shift of the proton on the anomeric carbon between pyridoxal phosphoglucose and glucose-1-P could be correlated with the expected changes in configuration at the anomeric position. Both NMR and optical rotation data indicate the synthesized pyridoxal phosphoglucose to be the β anomeric form.

RESULTS AND DISCUSSION

Apophosphorylase b from rabbit muscle can be reconstituted with a PLP derivative having a bulky substituent on the 5'-phosphate group (e.g. benzyl, phenylphosphoric, or pyridoxal-phosphoric group) without observable hindrance (6). The new PLP derivative, PLP-β-Glc, also bound to the apoenzyme accompanied by a CD spectral change as shown in Fig. 2. The CD titration experiment of the apoenzyme with PLP-β-Glc showed a stoichiometric binding. Incubation with an excess of PLP-β-Glc (up to 10 mol/apoenzyme monomer) resulted in no additional CD change. The CD spectrum of the PLP-β-Glc-bound enzyme was similar (but not the same) to those of the PLP-reconstituted enzyme as well as the intact holoenzyme. Since the two positive CD bands near 340 nm and 260 nm are assigned to a specific state of the enzyme-bound pyridoxal chromophore (14), both pyridoxal groups of PLP and PLP-β-Glc are suggested to bind to phosphorylase in a similar mode of binding and in a similar asymmetric environment to each other. Thus, PLP-β-Glc can occupy the original cofactor site.

The time course of the CD change at 335 nm induced by binding of PLP-β-Glc was apparently followed by a second order rate equation. The observed rate constant was 3.6 ± 0.4 M$^{-1}$s$^{-1}$ and independent of the molar ratio of PLP-β-Glc/apoenzyme (2.5 to 10). This value is much lower than the reconstitution rate constant for PLP (420 M$^{-1}$s$^{-1}$) obtained under the same conditions. As already was shown (6), the rate of reconstitution with the cofactor is markedly influenced by modification of the cofactor molecule; the rate decreases with the decrease in the number of anions at the 5' position of the cofactor and is apparently independent of the size of the 5' substituent, and the PLP derivatives having such an aromatic group as benzyl or phenyl group are more reactive. However, PLP-β-Glc shows the lowest reactivity (less than one order) toward reconstitution among the series of the PLP analogues containing a monovalent anion, i.e. 5'-monobenzyl phosphate, 5'-sulfate, 5'-monomethylphosphate, and 5'-deoxy-5'-sulfonate. This low reactivity would mean that since the site adjacent to the phosphate locus of the cofactor site has an affinity for a hydrophobic group rather than a hydrophilic group, the binding of PLP-β-Glc may be hindered by the force of exclusion of a hydrophilic group from a hydrophobic environment. A steric hindrance should also be considered since glucosyl group is more bulky than benzyl group.

Table I shows the enzyme activity of PLP-β-Glc-reconstituted phosphorylase b. If the PLP-β-Glc was a covalent intermediate in catalysis, the reconstituted enzyme would regain the enzyme activity following the cleavage of the glycosidic bond of PLP-β-Glc when the acceptor of glucosyl group was added. No enzyme activity was induced on incubation with both substrate, glycogen and glucose-1-P, and the allostERIC activator, AMP. It is possible to consider that the glucosyl group of PLP-β-Glc is bound in a wrong position for cleavage because the apoenzyme used was originally in the inactive b form. In order to test this possibility, the reconstitution ex-

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**Fig. 1.** 1H NMR spectrum at 100 MHz of PLP-β-Glc in D_2O. Inset, structural formula of PLP-β-Glc.

**Fig. 2.** CD spectra of rabbit muscle phosphorylase b. ---, Apoenzyme; - - - , PLP-reconstituted enzyme; --- , PLP-β-Glc-reconstituted enzyme. The reconstituted enzymes were obtained by incubation of 15 μM apoenzyme with 30 μM PLP or 29 μM PLP-β-Glc at 25°C for 24 h.

**Table I**

Effect of PLP-β-Glc on the activity of phosphorylase b

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cofactor</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holoenzyme, 10 μM</td>
<td>No addition</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+PLP-β-Glc, 200 μM</td>
<td>105</td>
</tr>
<tr>
<td>Apoenzyme, 10 μM</td>
<td>No addition</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>+PLP-β-Glc, 20 μM</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>+Lysate of PLP-β-Glc, 20 μM</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>+PLP, 20 μM</td>
<td>106</td>
</tr>
</tbody>
</table>

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Pyridoxal Phosphate in Phosphorylase

TABLE II
Exchange of enzyme-bound PLP-β-Glc by PLP

PLP-β-Glc enzyme was prepared by incubation of 10 μM apophosphorylase b with 20 μM PLP-β-Glc at 25°C for 24 h. The enzyme activity was measured after preincubation of 10 μM concentration of each enzyme with 200 μM PLP at 25°C for the indicated time. PLP-phosphorylase was also examined as a reference since a high concentration of PLP shows the inhibitory effect on the phosphorylase activity as was described by Avramovic-Zikic and Madsen (15).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme activity after incubation with an excess of PLP</th>
<th>μmol/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP-β-Glc enzyme</td>
<td>0 h</td>
<td>½ h</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>PLP enzyme</td>
<td>103</td>
<td>79</td>
</tr>
</tbody>
</table>

Experiments were carried out in the presence of several effectors of phosphorylase, such as 1 or 10 mM AMP, 10 mM MnCl₂, 0.1 mM P₄, 50 mM glucose-1-P, 0.1 mM maltopentaose, and 1 mg/ml of glycogen. Since proteins are structurally dynamic in solution, it would be expected that during the prolonged incubation of PLP-β-Glc-phosphorylase, breathing of protein allows the glucosyl group to fit into the correct position, resulting in a cleavage of the glycosidic bond. Both the single addition of the above effectors and the combination of 1 mM AMP plus each ligand to the reconstitution mixture, however, resulted in no increment of the activity of PLP-β-Glc-phosphorylase.

On the other hand, the prior acid hydrolysis of PLP-β-Glc caused reactivation of the apoenzyme to the same extent as the natural cofactor, PLP (Table I). On thin layer chromatography, the major spot of the lysate showed the same Rₓ value as PLP. This is coincident with the fact that the esteric bond of glucose-1-P is more labile against acid hydrolysis than the C-O-P bond of PLP.

Another possibility, i.e. that the irreversible inactivation by chemical modification of the nearby amino acid residue occurred via the cleavage of the bound PLP-β-Glc, was also examined. PLP-β-Glc-phosphorylase was, however, reactivated by incubation with an excess of PLP (Table II). This indicates that the enzyme-bound PLP-β-Glc could be exchanged with PLP to induce enzyme activity. The rate of exchange is slow but comparable to the rates of exchange of the other PLP analogues by PLP (6).

Tu et al. (16) have presented data supporting a glucosyl cation intermediate in the phosphorylase reaction in studies based on secondary isotope effect, while Firsov et al. (17) have obtained the results consistent with the covalent intermediate by the same technique. Even if the phosphorylase reaction proceeded via formation of a covalent glucosyl-enzyme intermediate, the present data would contradict the possibility of participation of the phosphate group of PLP in the covalent intermediate. Since Johnson et al. (5) have pointed out by x-ray crystallographic studies that there is no candidate for the nucleophilic group in acid base catalysis in phosphorylase except for the phosphate group of the cofactor, the present study would provide the evidence for the formation of a glucosyl cation intermediate in the phosphorylase reaction.

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REFERENCES