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Base-catalyzed reactivation of glucogen phosphorylase reconstituted with a coenzyme-substrate conjugate and its analogues

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Glycogen phosphorylase reconstituted with pyridoxal (5')diphospho(1)-α-D-glucose (PLDP-Glc) is catalytically inactive but slowly converted to the active enzyme through the cleavage of the pyrophosphate linkage. A similar reaction occurs more rapidly on PLDP-Gal and -Xyl but not on PLDP-Man. Values of pK_a for all the reactions are about 8.3, suggesting the participation of a common basic residue in these reactions. Based on the present and other results, it is presumed that Tyr-573 or Lys-574 acts as the base abstracting the proton from 2-hydroxyl group of the glucosyl moiety of PLDP-Glc.

Glycogen phosphorylase; Coenzyme-substrate conjugate; Pyridoxal 5'-phosphate; Enzyme mechanism

1. INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the reversible glucosyl transfer between glycogen and α-D-glucose 1-phosphate. The enzyme contains covalently bound PLP which is indispensable for catalysis [1]. We previously reported that the enzyme reconstituted with PLDP-Glc, a synthetic conjugate of PLP and Glc-1-P through a pyrophosphate linkage, is catalytically inactive but slowly converted to the active PLP-enzyme [2,3]. In the presence of glycogen, the glucosyl moiety of PLDP-Glc is transferred to the acceptor forming a new α-1,4-glucosidic linkage along with the production of the inactive PLDP-enzyme [3]. The glucosyl transfer from PLDP-Glc mimicks the normal catalytic reaction of phosphorylase not only in its action pattern but also in its kinetic properties [4]. These results provide evidence for the direct phosphate-phosphate interaction between PLP and Glc-1-P in the phosphorylase catalytic reaction.

We have more recently reported that the insertion of an additional phosphate between the two phosphate groups in PLDP-Glc is allowable for glucosyl transfer but not for the pyrophosphate cleavage [5]. The present study investigates the cleavage of pyrophosphate linkage in PLDP-Glc and its analogues having Gal, Man and Xyl in the place of Glc. The cleavage occurs more rapidly on PLDP-Gal and PLDP-Xyl than PLDP-Glc, but not on PLDP-Man. Values of pK_a for all the reactions are similar, about 8.3. No significant glycosyl transfers occur on any PLDP-sugars except for PLDP-Glc.

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Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; PLP, pyridoxal 5'-phosphate; PLDP, pyridoxal 5'-diphosphate; PLDP-Glc, -Gal, -Man and -Xyl, pyridoxal (5')diphospho(1)-α-D-glucose, -galactose, -mannose and -xylose, respectively; PLP-, PLDP-, PLDP-Glc-, PLDP-Gal-, PLDP-Man- and PLDP-Xyl-enzymes, enzymes reconstituted with the corresponding compounds; HPLC, high-performance liquid chromatography

2. EXPERIMENTAL

Rabbit muscle apophosphorylase *b* was prepared and

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reconstituted with any PLDP-sugar, as described in [5]. Assay of the enzyme activity was carried out in the direction of glycogen synthesis [6].

PLDP-Gal, -Man and -Xyl were synthesized from PLP and the corresponding sugar 1-phosphates essentially according to the method for the synthesis of PLDP-Glc [3]. All the procedures were carried out under reduced light. The lithium salts of PLDP-Gal and PLDP-Xyl having retention times on HPLC of 13.7 and 14.4 min were obtained in yields of 12 and 14%, respectively. The lithium salt of PLDP-Man having a retention time of 12.5 min was kept in an aqueous solution because of its high hygroscopy. PLDP-[^{14}C]Gal was synthesized in the same manner as described above, but on a reduced scale. The final product gave a single peak on HPLC as detected by absorbance at 324 nm and radioactivity. The concentration of the product was determined spectrophotometrically by using a molar absorption coefficient at 387 nm of 5000 $M^{-1} \cdot cm^{-1}$. PLDP and PLDP-Glc were synthesized according to the methods of Shimomura and Fukui [7] and Takagi et al. [3], respectively.

Pyridoxal compounds formed were reduced with sodium borohydride, and then analyzed with a C18 M & S pack column at room temperature on a Gilson liquid chromatography system at a flow rate of 0.4 ml/min. The solvent used was 50 mM potassium phosphate buffer (pH 6.4) containing 0.6 mM tetra-*n*-butylammonium bromide and 6% methanol. The absorbance at 324 nm of the effluent was monitored on a Variactor 311 UV detector, and the amount of pyridoxyl compound was determined by using system instruments chromatocorder 11.

3. RESULTS

When PLDP-Gal, -Man or -Xyl was mixed with rabbit muscle apophosphorylase *b* at pH 7.0 and 20°C, the molar ellipticity at 335 nm which is derived from the boud pyridoxal moiety was rapidly recovered to the level of the native holoenzyme. The second-order rate constants of the reconstitutions of PLDP-sugars with the apoenzyme at pH 7.0 and 20°C were determined from the rates of increase in molar ellipticity at 335 nm. The values for PLDP-Gal, -Man and -Xyl were 94, 52 and 100 $M^{-1} \cdot s^{-1}$, respectively, and are similar to or higher than that of PLDP-Glc under the same conditions (58 $M^{-1} \cdot s^{-1}$). Therefore, the position of each sugar moiety in those compounds might be suitably fitted to the active site pocket of the enzyme.

The reconstituted enzymes with PLDP-sugars were prepared by incubating a mixture of the apoenzyme with PLDP-sugar at pH 7.0 and 20°C for 24 h, except for PLDP-Xyl. The mixture containing PLDP-Xyl was kept at pH 7.0 and 0°C for 24 h, because a considerable increase in enzyme activity was observed during incubation at 20°C. The reconstitutions with any PLDP-sugar were completed under these conditions as judged by CD measurements. All the reconstituted enzymes showed little enzyme activities. The slight activities observed might be due to the trace contamination of PLP in the PLDP-sugars used and/or the reactivation during incubation. The reconstituted enzymes were incubated for a further three days at pH 6.7 or 8.3 in the absence or presence of glycogen, and enzyme activities were followed during incubation (fig.1). Enzyme activity of the PLDP-Glc-enzyme started to appear slowly, as observed previously [2]. The appearance of enzyme activity was more rapid at pH 8.3 than at pH 6.7, and completely repressed by the addition of glycogen. These results were explained by slow cleavage of the pyrophosphate linkage producing the active PLP-enzyme in the absence of glycogen, and, in the presence of glycogen, rapid transfer of the glucosyl moiety leaving the inactive PLDP-enzyme which is stable under the conditions used [2,3]. On the other hand, the reactivation of the PLDP-Gal and PLDP-Xyl-enzymes proceeded more rapidly than that of the PLDP-Glc-enzyme, being accelerated at a higher pH. Almost all of the original activity was recovered on the first day of incubation. However, no repressions in the reactivation by glycogen were observed for these two

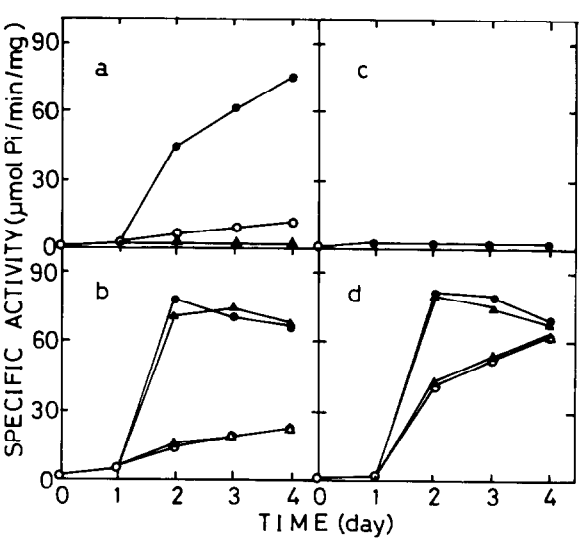

Fig.1. Reactivation of the enzymes reconstituted with PLDP-Glc (a), -Gal (b), -Man (c) and -Xyl (d). After one day of reconstitution, enzymes were incubated at pH 6.7 (○, △) or 8.3 (●, ▲) in the absence (○,●) or presence (△, ▲) of glycogen. The details are described in the text.

reconstituted enzymes, unlike for the PLDP-Glc-enzyme. In contrast with the reactivations of these three reconstituted enzymes, no reactivation was detected on the PLDP-Man-enzyme in the presence or absence of glycogen.

The reactivations of the PLDP-Glc-, PLDP-Gal- and PLDP-Xyl-enzymes were followed at various pH values. Rabbit muscle phosphorylase *b* is stable between pH 5 and pH 9.5 [8], and PLDP-Glc free in solution is also stable below pH 9. Fig.2 shows plots of the first-order rate constants observed (k_{obs}) against pH. From these plots, the first-order rate constant (k) and the dissociation constant (pK_a) were obtained and are summarized in table 1. The values of k greatly vary for different PLDP-sugars; the values for PLDP-Gal and PLDP-Xyl are 5 and 700 times as large as that for PLDP-Glc, respectively. In spite of the large difference in the k values, the values of pK_a for all of the compounds are in good agreement, about 8.3, suggesting the participation of a common basic group in these reactions.

The pyridoxal compound produced from enzyme-bound PLDP-Glc in the absence of glycogen was previously identified on ion-exchange chromatography as PLP [3]. The pyridoxal compounds produced from enzyme-bound PLDP-Gal and PLDP-Xyl were analyzed on HPLC. In the absence of glycogen, PLP was formed from PLDP-Gal (fig.3). The amounts of PLP formed at pH 6.7 and 8.3 correspond to the degrees of reactivation observed, indicating that the reactivation is due to the formation of the PLP-enzyme. Essentially the same results were obtained with the

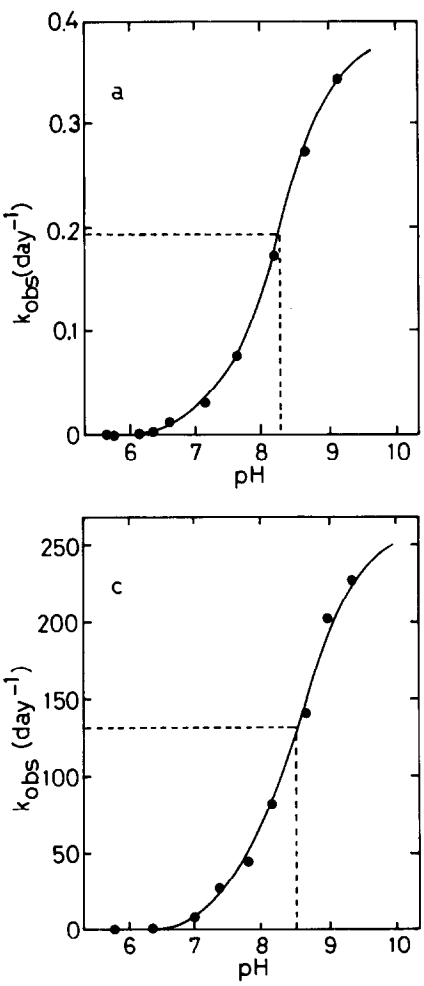

Fig.2. Effect of pH on the reactivation of PLDP-sugar-enzymes. The enzymes reconstituted with PLDP-Glc (a), -Gal (b) and -Xyl (c) were incubated in solutions containing 10 mM Mes, 10 mM Hepes, 10 mM triethanolamine, 20 mM 2-mercaptoethanol, and 0.1 M sodium chloride at various pH values at 20°C. Enzyme activities were determined at various time intervals by taking aliquots of the incubation mixture, and first-order rate constants (k_{obs}) were calculated from the enzyme activities determined. The solid lines represent theoretical curves that most fit the data obtained.

Table 1

Kinetic parameters for the reactivation of the PLDP-sugar-enzyme

	k (day^{-1})	pK_a
PLDP-Glc	0.38	8.2
PLDP-Gal	1.8	8.3
PLDP-Xyl	260	8.5

PLDP-Xyl-enzyme as well as the PLDP-Glc-enzyme.

In the presence of glycogen, formation of small amounts of PLDP from enzyme-bound PLDP-Gal and PLDP-Man was observed, but not from PLDP-Xyl (not shown). The rates of PLDP formation from PLDP-Gal and -Man were approx. 1/3000 and far below 1/10000, respectively, as compared with that from PLDP-Glc. In a separate experiment, a slight but significant incorporation of ^{14}C from enzyme-bound PLDP-[^{14}C]Gal was observed. A similar rate of galactosyl transfer from α-D-galactose 1-phosphate, however, was also observed using the native enzyme, suggesting that the galactosyl transfer from PLDP-Gal is due to the inherent looseness of the enzyme in the specificity for sugar moieties.

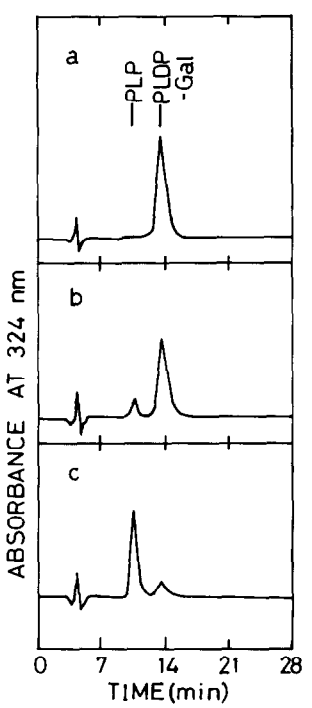

Fig.3. Analysis on HPLC of pyridoxal compounds formed from enzyme-bound PLDP-Gal in the absence of glycogen. (a) Zero-time control; (b) after 24 h at pH 6.7; (c) after 24 h at pH 8.3. The experimental details are described in the text.

4. DISCUSSION

The mechanism for the cleavage of the pyrophosphate linkage in the enzyme-bound PLDP-sugars are presumed on the analogy of the base-catalyzed degradation of UDP-Glc [9]; the reaction is started by the abstraction of a proton from the 2-hydroxyl group of the sugar moiety by a base producing sugar 1,2-cyclic phosphate and the PLP-enzyme. The fact that this type of reaction does not occur on PLDP-Man, which has a different configuration of the 2-hydroxyl group, strongly supports the proposed mechanism. In PLDP-Man, the base group might be unable to abstract a proton from the 2-hydroxyl group, or, if this occurred, the oxygen anion formed might be unable to attack the phosphorus atom in the neighboring phosphate group. Based on the three-dimensional structure of the active site in rabbit muscle phosphorylase *b* [10] as well as the value of pK_a, the most probable candidate for the basic group is Tyr-573 (according to the revised numbering of this enzyme [11]). Lys-574 might be another candidate for this group, although lysine has normally a higher pK_a than Tyr. Since the glucosyl moiety of PLDP-Glc might occupy the same site in the enzyme as that of Glc-1-P, either one of these residues should be located close to the 2-hydroxyl group of the substrate.

The cleavage of the pyrophosphate linkage in PLDP-Gal and PLDP-Xyl proceeds 5 and 700 times more rapidly than that of PLDP-Glc, respectively. This could result from better orientation of the sugar moieties of PLDP-Gal and PLDP-Xyl to the basic group than that of PLDP-Glc. Street et al. [12] have recently reported the order in the strength of hydrogen bonds between each position on the glucosyl moiety and the surrounding in rabbit muscle phosphorylase to be $6 \approx 3 > 4 > 2 \approx 1$. According to this order, the xylosyl moiety, lacking a hydroxymethyl group on C5, is the most strongly affected within a group of sugars analogous to glucose. The galactosyl moiety, having an alternate configuration on C4, is less affected

upon binding in the network of hydrogen bondings in the active site pocket. Thus, the xylosyl moiety might easily orient to the basic group, so that PLDP-Xyl is subjected to the most rapid cleavage within the PLDP-sugars tested.

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