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Catalytic mechanism of glycogen phosphorylase: Pyridoxal(5′)diphospho(1)-α-D-glucose as a transition-state analogue

(pyridoxal 5′-phosphate/pyridoxal 5′-diphosphate/glucosyl transfer/phosphate–phosphate interaction)

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ABSTRACT **Pyridoxal(5′)diphospho(1)-α-D-glucose was used to reconstitute glycogen phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) from rabbit muscle, replacing the natural pyridoxal 5′-phosphate coenzyme. Incubation of the reconstituted enzyme alone resulted in the gradual cleavage of the synthetic cofactor to pyridoxal 5′-phosphate, which caused slow reactivation of the enzyme. The addition of maltopentaose or glycogen altered the mode of cleavage; the cofactor was rapidly decomposed to pyridoxal 5′-diphosphate. The radioactive glucose moiety released from pyridoxal(5′)diphospho(1)-α-D-[^{14}C]glucose was incorporated into the outer chain of glycogen, forming an α-1,4-glucosidic linkage. These results show that the glucosyl transfer reaction discovered mimics the normal catalysis of this enzyme, and they strongly support the catalytic mechanism in which the coenzyme phosphate acts as a catalyst by direct interaction with the phosphate of the substrate, forming the pyrophosphate-like transition intermediate.**

The catalytic mechanism of glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) remains unclear despite intensive investigations. However, evidence has been accumulating supporting the catalytic function of pyridoxal 5′-phosphate (PL*P*) bound to this enzyme: (*i*) The amino acid sequences around the cofactor-linked residues are highly conserved in phosphorylases from many sources (1–4). (*ii*) There is a large enough space to allow the binding of a bulky group adjacent to the phosphate group of the cofactor, which interacts with positive charges (5, 6). (*iii*) All the substrates bind near the cofactor, and the phosphate groups of α-D-glucose 1-phosphate (Glc-1-*P*) and PL*P* are located close together (7–9). The phosphate group of PL*P* is likely to be involved in the catalytic mechanism.

On the basis of the results of ^{31}P NMR studies, Withers *et al.* (10) suggested that the phosphorus of the constrained dianion of the coenzyme could be an electrophile which, by direct interaction with the phosphate group of Glc-1-*P*, would facilitate the breakage of the glucosidic linkage. This hypothesis was substantiated by the recent investigation carried out collaboratively by the Edmonton group and ourselves (11). Rabbit muscle phosphorylase *b* reconstituted with pyridoxal(5′)diphospho(1)-α-D-glucose (PL*PP*-α-Glc) demonstrated no enzyme activity but was slowly reactivated on prolonged incubation. The reactivation was markedly diminished in the presence of maltopentaose or glycogen. The ^{31}P NMR spectrum of PL*PP*-α-Glc bound to the enzyme showed that the phosphate group was constrained by positive charges. The addition of maltopentaose, a glycogen analogue and an alternative substrate, resulted in the disappearance of this spectrum and its replacement with one characteristic of pyridoxal 5′-diphosphate (PL*PP*) bound to the enzyme. Therefore, glucose was released from the synthetic coenzyme.

This paper describes the results of the identification of pyridoxal compounds produced from the enzyme-bound PL*PP*-α-Glc under various conditions as well as the fate of the radioactive glucose released from the PL*PP*-α-[^{14}C]Glc in the presence of glycogen. The glucose was recovered in the outer chain of glycogen, bound through an α-1,4-glucosidic linkage. The glucosyl transfer reaction discovered mimics the normal catalysis of this enzyme, supporting strongly the catalytic mechanism based on the direct phosphate–phosphate interaction between PL*P* and the substrate.

MATERIALS AND METHODS

Maltose, isomaltose, and oyster glycogen were purchased from Nakarai Chemicals (Kyoto, Japan), and maltopentaose was from Seishin Pharmaceutical (Tokyo, Japan). Kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) and nigerose (3-*O*-α-D-glucopyranosyl-D-glucose) octaacetate were kindly donated by Kazuo Matsuda (Tohoku University, Sendai, Japan). Nigerose was obtained by deacetylation of the octaacetyl derivative with sodium methylate. PL*PP* was synthesized as reported (5). Resolution and reconstitution of rabbit muscle phosphorylase *b* with PL*PP*-α-Glc were carried out essentially as described (11). Radioactivity was measured in a Beckman LS-9000 liquid scintillation system.

Synthesis of PL*PP*-α-Glc. PLP monohydrate (0.53 g, 2 mmol) was dissolved in 20 ml of chloroform containing 0.28 ml of triethylamine. After evaporation, the residue was redissolved in 15 ml of chloroform containing 0.4 ml of triethylamine. Diphenyl phosphochloridate (0.52 ml, 2.5 mmol) was added with stirring, and then the solution was kept at room temperature for 3 hr under anhydrous conditions. The yellow syrup obtained after evaporation was shaken with dry diethyl ether to remove the excess diphenyl phosphochloridate, and then the ether was removed by decantation and the residue was dried under reduced pressure. To this syrup was added tri-1-butylammonium Glc-1-*P* (6 mmol) in 10 ml of dry pyridine. After being left overnight at room temperature, the solution was evaporated to remove the pyridine. The residue was dissolved in 20 ml of water, and the solution was extracted with 20 ml of chloroform and then with 20 ml of diethyl ether. The aqueous layer diluted with 500 ml of water was applied on a Dowex 1-X8 (Cl^-) column (1.8 × 17 cm) and eluted successively with 200 ml of water, 450

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Abbreviations: Glc-1-*P*, α-D-glucose 1-phosphate; PL*P*, pyridoxal 5′-phosphate; PL*PP*, pyridoxal 5′-diphosphate; PL*PP*-α-Glc, pyridoxal(5′)diphospho(1)-α-D-glucose.

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ml of 0.01 M HCl/0.0125 M LiCl, and 500 ml of 0.01 M HCl/0.017 M LiCl. Fractions (20 ml) were collected and absorbance at 390 nm was measured by taking 0.1-ml aliquots after dilution with 3 ml of 0.1 M NaOH. The compounds eluted were identified by thin-layer chromatography. Fractions containing PL*PP*-α-Glc were combined and neutralized with 1 M LiOH. The lithium salt of PL*PP*-α-Glc in the concentrated solution was precipitated by addition of acetone/methanol, 4:1 (vol/vol), and converted into the sodium salt with the aid of Dowex 50W-X8 (H^+ form). The sodium salt of PL*PP*-α-Glc in the concentrated aqueous solution was precipitated with acetone and identified as described (11).

PL*PP*-α-[^{14}C]Glc was synthesized from PL*P* and [^{14}C]Glc-1-*P* (New England Nuclear, 294 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) according to the above procedure but in a 1/600 scale, and isolated after mixing with unlabeled PL*PP*-α-Glc. Special care was taken during the isolation to avoid hydrolysis of the product. The final product gave a single spot on thin-layer chromatography when detected by fluorescence and radioactivity.

RESULTS

Cleavage of PL*PP*-α-Glc Bound to Phosphorylase. PL*PP*-α-Glc bound to rabbit muscle phosphorylase *b* is cleaved slowly to produce PL*P* and, in the presence of glycogen or maltopentaose, more rapidly to produce PL*PP*. This conclusion has been drawn from the results of reactivation and ^{31}P NMR analysis of the reconstituted enzyme after incubation under different conditions (11). To confirm this mode of cleavage, the pyridoxal compounds formed were analyzed by Dowex 1 column chromatography. During incubation of the reconstitution mixture at pH 6.6 for 3 days, the PL*PP*-α-Glc remained mostly unchanged, but a small amount of PL*P* was formed (Fig. 1*A*). The amount of PL*P* formed corresponded to the appearance of enzyme activity during incubation (11). A much larger amount of PL*P* was formed from the same reconstitution mixture incubated at pH 7.9 (Fig. 1*B*), in agreement with the accelerated reactivation at a higher pH (11). In the presence of maltopentaose, PL*PP*-α-Glc was almost exclusively converted to PL*PP* (Fig. 1*C*). Because PL*PP* does not activate apophosphorylase (5), the repressed reactivation observed (11) might be due to the small amount of PL*P* that had been formed before addition of maltopentaose. Incubation of the reconstitution mixture in the presence of maltopentaose at pH 6.5 or 7.8 for 1 or 24 hr gave essentially the same results (data not shown).

Fate of the Glucose Moiety Liberated from PL*PP*-α-Glc. PL*PP*-α-Glc bound to phosphorylase was decomposed to PL*PP* in the presence of maltopentaose. Therefore, the glucose moiety was lost from PL*PP*-α-Glc. Its fate was determined by using PL*PP*-α-[^{14}C]Glc. Maltopentaose was replaced by glycogen because of the ease in separating it from the reaction mixture. Fig. 2 shows the time course of radioactivity incorporation into glycogen and other fractions. The radioactive glucose was rapidly incorporated into glycogen, and the incorporation reached 78% of the original amount of PL*PP*-α-[^{14}C]Glc after 1 hr. Some radioactivity was found in the protein fraction. However, even the zero-time control contained a considerable amount of radioactivity. This might be due to incomplete separation of PL*PP*-α-[^{14}C]Glc from the enzyme or the unspecific binding of aldehyde to protein.

The glucosyl transfer reaction proceeded much more rapidly than the reactivation in the absence of carbohydrate and coincided with the rapid conversion of PL*PP*-α-Glc to PL*PP* in the presence of maltopentaose. The apparent $t_{1/2}$ of incorporation of the radioactive glucose moiety into glycogen under these conditions was 13 min.

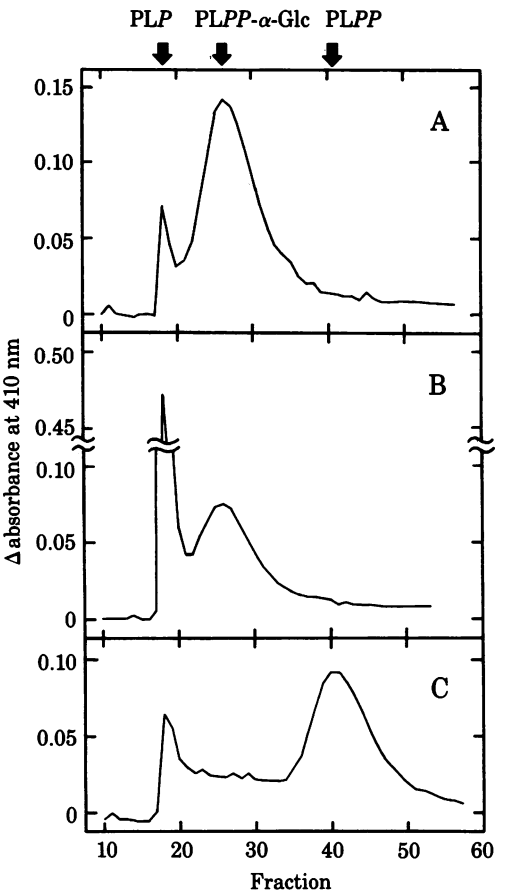

FIG. 1. Analyses of the pyridoxal compounds formed from the enzyme-bound PL*PP*-α-Glc. The apoenzyme (208 μM) was mixed with 230 μM PL*PP*-α-Glc in 0.1 M sodium glycerophosphate, pH 6.5/20 mM mercaptoethanol, and the mixture was incubated at 25°C. After 20 hr, the mixture was passed through a Sephadex G-25 column (1.2 × 26 cm) equilibrated with 0.1 M NaCl/10 mM 2-(*N*-morpholino)ethanesulfonic acid/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/10 mM triethanolamine/20 mM mercaptoethanol, pH 6.8, and eluted with the same solution. The protein solution (188 μM) was divided into four portions, and each was mixed with 0.1 M triethanolamine (adjusted to different pH values)/20 mM mercaptoethanol and 1 mM AMP with or without maltopentaose. After measurement of pH, the mixtures were incubated at 25°C. At the periods of 1 and 24 hr, the solutions were cooled to 0°C and each was mixed with 2 ml of 5% cold trichloroacetic acid. After centrifugation, the precipitates were each washed twice with 2 ml of cold 2.5% trichloroacetic acid. The supernatants and washings were combined and extracted with diethyl ether. The neutralized solutions were separately applied on a column (0.5 × 10 cm) of Dowex 1-X8 (Cl^-, 200–400 mesh). The column was first washed with 10 ml of water and then pyridoxal compounds were eluted by a linear gradient of 2 mM HCl/NaCl (10 to 55 mM, 25 ml each). Fractions (2 ml) were collected and the pyridoxal contents were determined according to the method of Wada and Snell (12). (*A*) pH 6.5 without maltopentaose. (*B*) pH 7.8 without maltopentaose. (*C*) pH 6.5 with 50 mM maltopentaose. The specific activities of the reconstituted enzymes after the incubation under conditions of *A–C* were 6.5, 41.4, and 2.7 μmol/min per mg of protein, respectively. The apoenzyme used had a specific activity of 0.6 μmol/min per mg of protein.

Mode of Binding of the Glucose Moiety to Glycogen. To determine the binding mode of the glucose moiety with glycogen, the radioactive glycogen produced was digested with β-amylase and the digest was analyzed by paper chromatography. The radioactive materials before and after the digestion were, respectively, found at the origin and the position of maltose (α-1,4), which is distinguishable from that of isomaltose (α-1,6) (Fig. 3 *A* and *B*). Treatment of the β-amylase lysate with α-glu-

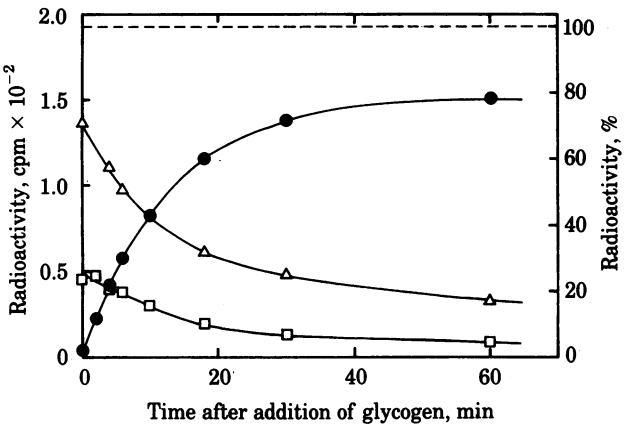

FIG. 2. Fate of the radioactive glucose moiety from the enzyme-bound PL*PP*-α-[^{14}C]Glc. The apoenzyme (91.7 μM) was mixed with 65.2 μM PL*PP*-α-[^{14}C]Glc (1.86 × 10^5 cpm/μmol) in 0.1 M NaCl/10 mM 2-(*N*-morpholino)ethanesulfonic acid/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid/10 mM triethanolamine/20 mM mercaptoethanol, pH 6.8, and the mixture was incubated at 25°C. After 1 day, twice the volume of glycogen at 9.1 mg/ml was added to the mixture, and incubation was continued at 25°C. At the indicated time intervals, 0.5-ml portions were taken and heated at 90°C for 2.5 min. The precipitates formed were collected by centrifugation and washed with 1 ml of water. To the combined solution of the supernatant and washing was added 2.1 ml of cold ethanol. The mixture was centrifuged at 0°C to obtain the precipitate. The heat precipitate was suspended in 0.1 M NaOH and mixed with Aquasol-2 (New England Nuclear). After neutralization, the radioactivity was measured (protein fraction, □). The ethanol precipitate was dissolved in water and its radioactivity was measured (glycogen fraction, ●). A portion of the ethanol supernatant was used for measurement of radioactivity (supernatant fraction, △). Values of the zero-time control were obtained with the heated enzyme solution.

cosidase followed by paper chromatography converted all the radioactivity to the position of glucose (Fig. 3*C*), providing the evidence for the binding of the radioactive glucose through an α-glucosidic linkage. However, maltose was indistinguishable from nigerose (α-1,3) and kojibiose (α-1,2) under the above chromatographic conditions. The presumptive maltose was compared with these three glucobioses by paper chromatography under different conditions and was found to migrate coincidently with maltose but differently from nigerose and kojibiose (Fig. 3*D*). These results indicate unequivocally that the glucose moiety of PL*PP*-α-Glc was transferred to the outer chain of glycogen, forming a new α-1,4-glucosidic linkage.

DISCUSSION

PL*PP*-α-Glc is a compound that combines the phosphates of the coenzyme PL*P* and the substrate Glc-1-*P* in a pyrophosphate linkage. When bound to the enzyme, this compound is slowly cleaved to produce PL*P*, which activates the enzyme. The addition of glycogen (or maltose) altered the mode of cleavage, giving rise to the rapid formation of PL*PP* and the simultaneous transfer of the glucose moiety to the outer chain of glycogen. The glucose moiety transferred is bound through an α-1,4-glucosidic linkage. Therefore, this glucosyl transfer reaction exactly mimics the normal catalysis of the enzyme, providing evidence for the direct phosphate–phosphate interaction between PL*P* and Glc-1-*P* in the normal catalytic mechanism, as postulated previously (11).

This mechanism requires the close approach of the two phosphates on the enzyme. X-ray crystallographic data showed the phosphate of the coenzyme was 7 Å from the phosphate of Glc-1-*P* (13, 14). Jenkins *et al.* (15) have recently reported that glu-

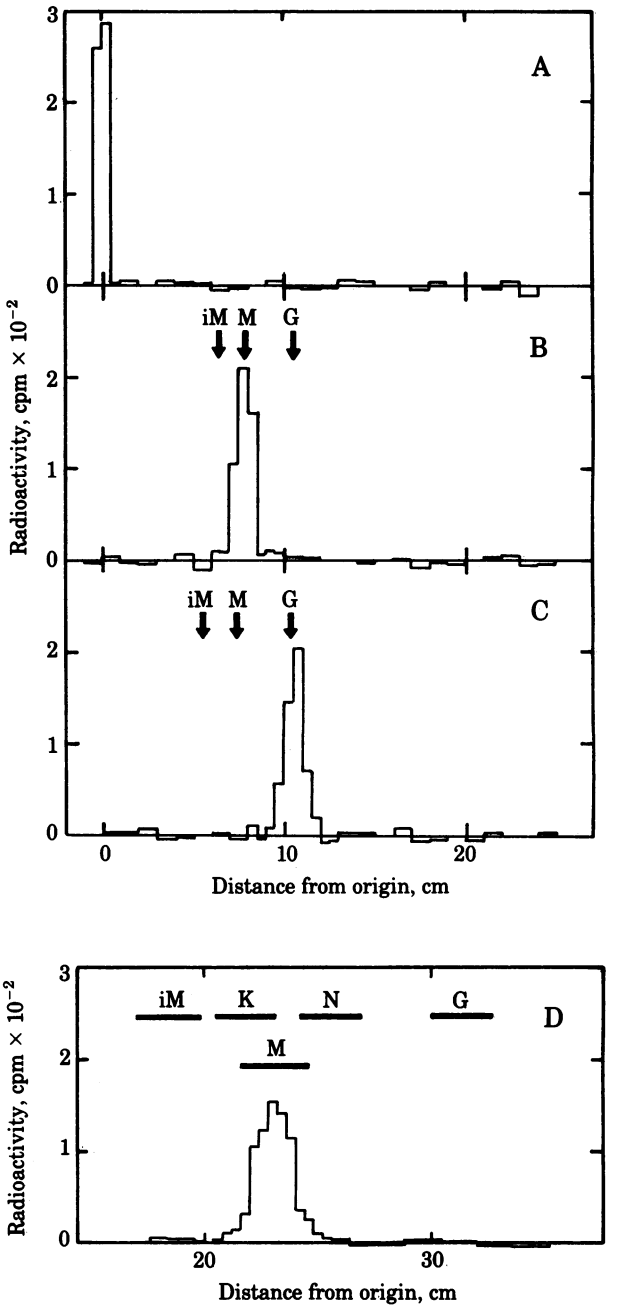

FIG. 3. Paper chromatographic analyses of digests of the radioactive glycogen with β-amylase and α-glucosidase. Radioactive glycogen (1.8 mg, 1.4 × 10^4 cpm) was obtained by incubation of the PL*PP*-α-[^{14}C]Glc-reconstituted enzyme with glycogen and dissolved in 0.2 ml of water. (*A*) A 10-μl portion of the glycogen solution was chromatographed without digestion. (*B*) An 80-μl portion was digested with β-amylase (Boehringer Mannheim, 0.5 unit) in 10 mM ammonium acetate, pH 4.8, at 37°C for 60 min. The lyophilized digest was dissolved in water and used for chromatography. (*C*) The lyophilized β-amylase lysate was dissolved in 10 mM ammonium acetate, pH 6.0, and digested with α-glucosidase (Boehringer Mannheim, 2 units) at 30°C for 3.5 hr. The lyophilized digest was dissolved in water and used for chromatography. Ascending paper chromatography of *A–C* was carried out on Whatman 3MM paper with a solvent system of 1-butanol/pyridine/water, 6:4:3 (vol/vol), at room temperature for 17 hr. (*D*) The β-amylase lysate was spotted on Toyo no. 51A paper and chromatographed in a descending manner with a solvent system of 1-butanol/pyridine/water, 6:4:3 (vol/vol), at room temperature for 59 hr. The paper strips were cut into pieces 0.4–1.6 cm wide and the radioactivity was determined in a toluene-based scintillator. G, glucose; M, maltose; iM, isomaltose; K, kojibiose; N, nigerose.

cose 1,2-cyclic phosphate is in almost the same position on the enzyme as Glc-1-*P* is, whereas the phosphate of the former is only 3.8 Å from the phosphate of the coenzyme. Shimomura and Fukui (5) showed that PL*PP* binds to apophosphorylase at a rate much higher than that for PL*P*, indicating a strong binding site for such a pyrophosphate moiety. Parrish *et al.* (16) suggested that a single molecule of inorganic pyrophosphate was capable of simultaneously occupying both the coenzyme and substrate phosphate binding pockets.

Binding of the two phosphate groups close together would lead to undesirable electrostatic repulsion. This difficulty could be minimized by neutralization of some of the charges, either by direct protonation or by tight coordination of one of the phosphates with nearby basic groups. The presence of such basic groups has been shown in various studies. Shimomura *et al.* (17) showed in affinity labeling studies that the ε-amino group of Lys-573 was located close to PL*P*. The results of chemical modification studies by Dreyfus *et al.* (18) and of inhibition studies by Miller *et al.* (19) suggested that Arg-568 was involved in the active site. The sequence around these residues contains many basic residues, including two Lys-Arg sequences (1), and x-ray crystallographic studies (13, 14) revealed that this region was near the phosphate groups of the coenzyme and the substrate.

Fig. 4 illustrates the proposed role of the coenzyme phosphate in the catalytic mechanism of phosphorylase, compared with the chemical structure of PL*PP*-α-Glc bound to the enzyme. Positive charges in the active site may constrain the coenzyme phosphate into a configuration that confers electrophilicity upon the phosphorus. This facilitates nucleophilic attack by the phosphate of Glc-1-*P* and cleavage of the glucosidic linkage. The mechanism for the following transfer remains to be studied, although Helmreich and Klein (20) showed the incorporation of radioactivity from [^{14}C]Glc-1-*P* into potato phosphorylase in the presence of cyclodextrin.

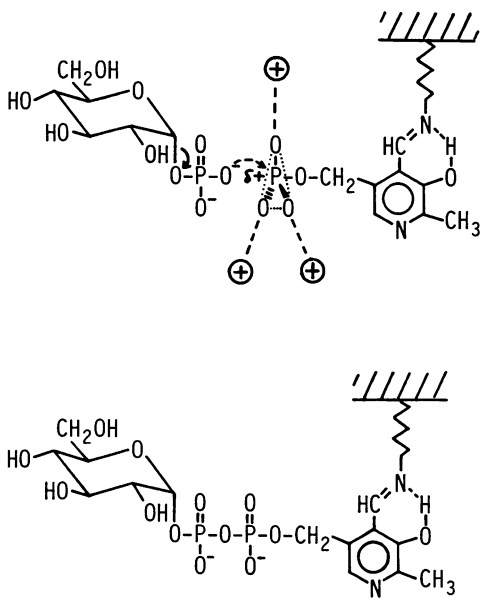

FIG. 4. Proposed catalytic mechanism of glycogen phosphorylase (*Upper*) and the structure of PL*PP*-α-Glc bound to the enzyme (*Lower*). ⊕, Positive charges or hydrogen-bond donors.

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1. Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Kumar, S., Wade, R. D., Walsh, K. A., Neurath, H. & Fischer, E. H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4762–4766.
2. Lerch, K. & Fischer, E. H. (1975) *Biochemistry* **14,** 2009–2014.
3. Nakano, K., Wakabayashi, S., Hase, T., Matsubara, H. & Fukui, T. (1978) *J. Biochem.* **83,** 1085–1094.
4. Schächtele, K. H., Schiltz, E. & Palm, D. (1978) *Eur. J. Biochem.* **92,** 427–435.
5. Shimomura, S. & Fukui, T. (1978) *Biochemistry* **17,** 5359–5367.
6. Shimomura, S., Emman, K. & Fukui, T. (1980) *J. Biochem.* **87,** 1043–1052.
7. Sygusch, J., Madsen, N. B., Kasvinsky, P. J. & Fletterick, R. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4757–4761.
8. Weber, I. T., Johnson, L. N., Wilson, K. S., Yeates, D. G., Wild, D. L. & Jenkins, J. A. (1978) *Nature (London)* **274,** 433–437.
9. Shimomura, S. & Fukui, T. (1980) *Biochemistry* **19,** 2287–2294.
10. Withers, S. G., Madsen, N. B. & Sykes, B. D. (1981) *Biochemistry* **20,** 1748–1756.
11. Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S. & Fukui, T. (1981) *J. Biol. Chem.* **256,** 10759–10762.
12. Wada, H. & Snell, E. E. (1961) *J. Biol. Chem.* **236,** 2089–2095.
13. Sprang, S. & Fletterick, R. J. (1979) *J. Mol. Biol.* **131,** 523–551.
14. Johnson, L. N., Jenkins, J. A., Wilson, K. S., Stura, E. A. & Zanotti, G. (1980) *J. Mol. Biol.* **140,** 565–580.
15. Jenkins, J. A., Johnson, L. N., Stuart, D. I., Stura, E. A., Wilson, K. S. & Zanotti, G. (1981) *Philos. Trans. R. Soc. London. Ser. B* **293,** 23–41.
16. Parrish, R. F., Uhing, R. J. & Graves, D. J. (1977) *Biochemistry* **16,** 4824–4831.
17. Shimomura, S., Nakano, K. & Fukui, T. (1978) *Biochem. Biophys. Res. Commun.* **82,** 462–468.
18. Dreyfus, M., Vandenbunder, B. & Buc, H. (1980) *Biochemistry* **19,** 3634–3642.
19. Miller, J. F., Seybold, M. C. & Graves, D. J. (1981) *Biochemistry* **20,** 4579–4584.
20. Helmreich, E. J. M. & Klein, H. W. (1980) *Angew. Chem. Int. Ed. Engl.* **19,** 441–455.