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Does Pyridoxal 5′-Phosphate Function in Glycogen Phosphorylase as an Electrophilic or a General Acid Catalyst?†

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ABSTRACT: α-D-Glucose 1-diphosphate interacts with pyridoxal-reconstituted rabbit muscle phosphorylase *b* activated by AMP (AMP-S). Under these conditions, the glucose moiety of α-D-[^{14}C]glucose 1-diphosphate is transferred to limit dextrin forming α(1→4) glycosidic bonds and simultaneously releasing pyrophosphate as shown by ^{31}P NMR spectroscopy. Thus, specific structural requirements invoked to explain the reactions of pyridoxal(5′)diphospho(1)-α-D-glucose need not to be assumed in the case of the reactions of α-D-glucose 1-diphosphate. Dianions isomorphous to phosphate activate pyridoxal phosphorylase regardless of their p*K* values while the same anions, when bound covalently to pyridoxal, are inactive. Thus, anions bound noncovalently to pyridoxal phosphorylase act differently than anions linked covalently to pyridoxal, such as the 5′-phosphate group of pyridoxal 5′-phosphate, which is postulated to be part of a proton donor–acceptor pathway. The reaction of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (heptenitol) with phosphorylase yields, in the presence of orthophosphate as a glycosyl acceptor, 1-deoxy-D-*gluco*-heptulose 2-phosphate (heptulose-2-P). This sugar phosphate is unreactive but a potent competitive inhibitor for rabbit muscle phosphorylase *b* and potato phosphorylase with respect to α-D-glucose 1-phosphate: $K_i = 14 \times 10^{-6}$ M and 1.9×10^{-6} M, respectively. Heptulose-2-P is ideally suited for ^{31}P NMR experiments with phosphorylase because its phosphate resonance and that of the 5′-phosphate of pyridoxal 5′-phosphate do not overlap, and in contrast to α-D-glucopyranose cyclic 1,2-phosphate, another powerful inhibitor of glycogen phosphorylases, heptulose-2-P is protonatable in the pH range of the enzymatic reaction. Use was made of this property of heptulose-2-P in ^{31}P NMR investigations, the results of which indicated that the dianionic 5′-phosphate group of the natural cofactor becomes partially protonated on binding of heptulose-2-P to potato phosphorylase, whereas the monoprotonated 5′-phosphonate group of the partially active 5′-deoxypyridoxal-5′-(methylenephosphonate) muscle phosphorylase *b* derivative was shown to share a proton with the phosphate moiety of heptulose-2-P. While the experiments support a role for the cofactor phosphate in glycogen phosphorylases in a general acid–base catalysis [cf. Feldmann, K., Hörl, M., Klein, H. W., & Helmreich, E. J. M. (1978) *Proc. FEBS Meet. 42*, 205–218], they do not uphold a function of the phosphorus of the 5′-phosphate group of the cofactor as an electrophilic catalyst [cf. Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S., & Fukui, T. (1981) *J. Biol. Chem. 256*, 10759–10762].

Our preceding studies of phosphorylase reactions with glycosylic[1] substrates such as D-glucal, heptenitol, and glucosyl fluoride indicated that α-glucan phosphorylases are general acid–base catalysts requiring, in addition to suitably charged amino acid side chains, phosphate (arsenate) and a 5′-phosphate anion linked covalently to pyridoxal (Klein et al., 1982, 1984; Palm et al., 1983). On the basis of these experiments, a role for the 5′-phosphate group of the cofactor as a general acid was proposed. In disagreement with this hypothesis, Madsen and colleagues (Withers et al., 1981b, 1982a; Takagi

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[1] According to Hehre et al. (1980), glycosylic substrates are compounds that yield a glycosyl residue on protonation.

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et al., 1982) have postulated a direct interaction between the phosphorus of the 5′-phosphate group of pyridoxal-P[2] and the oxygen of the substrate phosphate. Phosphate–phosphate interactions were also implicated by the experiments of Parrish et al. (1977) demonstrating activation of pyridoxal phosphorylase by phosphate and inhibition by pyrophosphate.

The inhibition by pyrophosphate was thought to involve competition for two adjacent phosphate binding sites: the site normally occupied by the phosphate covalently linked to the cofactor and the phosphate subsite of glucose-1-P. In keeping with the idea of direct phosphate–phosphate interactions, Withers et al. (1981a) have interpreted ^{31}P NMR spectra of the 5′-phosphate of pyridoxal-P in muscle phosphorylases *b* and *a* in the presence of maltopentaose and the inhibitor glucose-1,2-P as indicating the existence of the cofactor phosphate as dianion, which in the course of activation becomes tightly coordinated by interaction with neighboring basic groups and oriented in a trigonal-bipyramidal configuration with the empty apical position pointing toward the substrate phosphate. This should allow the coenzyme phosphorus to act as an electrophilic catalyst that labilizes the phosphate ester bond of glucose-1-P. Formation of a pseudo pyrophosphate bond was thought to be the committed step in catalysis (Withers et al., 1981b, 1982a; Takagi et al., 1982). The fact that pyridoxal-PP-α-glucose was reactive with muscle phosphorylase and was shown to transfer its glucosyl residue to a saccharide acceptor releasing pyridoxal-PP was considered to support this mechanism (Takagi et al., 1982). We now show that PP-α-glucose is also reactive with a pyridoxal-reconstituted muscle phosphorylase and that the rates of glucosyl transfer with this compound and pyridoxal-PP-α-glucose are comparable.

The assignment of the phosphorus of the cofactor as an immobilized dianion by ^{31}P NMR was based on experiments with the inhibitor glucose-1,2-P (Withers et al., 1981a). The fact that this cyclic sugar phosphate is unprotonatable in the pH range where phosphorylase is active, however, limits its usefulness. We have therefore used heptulose-2-P as inhibitor. Heptulose-2-P, unlike glucose-1,2-P but like glucose-1-P, is a monophosphate ester protonatable in the pH range of the phosphorylase reaction. Heptulose-2-P was shown before to be a dead-end product of the reaction of phosphorylase with heptenitol and P_i (Klein et al., 1984). It turned out to be the most potent inhibitor known for the phosphorylase reaction with glucose-1-P. Moreover, the ^{31}P resonance signal of heptulose-2-P is shifted to higher fields far enough not to interfere with the phosphate resonance of pyridoxal-P. Therefore, ^{31}P NMR studies were carried out with heptulose-2-P. Additional data were collected with a rabbit muscle phosphorylase *b* derivative containing the cofactor analogue 5′-deoxypyridoxal-5′-(methylenephosphonate). This is the only known cofactor analogue covalently modified at the 5′-position that is active when bound to muscle phosphorylase. This phosphorylase derivative has one-fourth the activity of the natural pyridoxal-P-containing enzyme (Vidgoff et al., 1974). Like pyridoxal phosphorylase *b*, the 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase *b* derivative binds the allosteric activator 5′-AMP 10 times more tightly than the natural pyridoxal-P-containing enzyme (Vidgoff et al., 1974), suggesting that both phosphorylase derivatives are in the *R* conformation [cf. Withers et al. (1982b)]. Moreover, the 5′-deoxypyridoxal 5′-(methylenephosphonate) phosphorylase *b* derivative has a pH activity dependence not much different from that of the natural pyridoxal-P-containing enzyme (Vidgoff et al., 1974). Finally, the resonance of the phosphonate group of the 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase *b* derivative is well separated from the phosphate resonance of heptulose-2-P, and most importantly, the pH-dependent chemical shift of the 5′-deoxypyridoxal-5′-(methylenephosphonate) Schiff base measured by ^{31}P NMR spectroscopy is in the opposite direction to that for the corresponding pyridoxal-P Schiff base (Schnackerz & Feldmann, 1980).

The results of the experiments with heptulose-2-P, potato phosphorylase, and the 5′-(methylenephosphonate)-containing muscle phosphorylase together with a reconsideration of the activities of pyridoxal phosphorylase *b* with PP-α-glucose and anions such as fluorophosphate with pK_{II} too low to function as a general acid catalyst reinforce our previous arguments (Klein et al., 1982, 1984) against a participation of the phosphorus of pyridoxal 5′-phosphate as an electrophile in phosphorylase catalysis, as proposed by Madsen and his colleagues (Withers et al., 1981a,b, 1982a; Takagi et al., 1982).

Experimental Procedures

Reagents. Glucose-1-P, AMP-S, and AMP were from Boehringer, Mannheim, West Germany. Maltotetraose and maltoheptaose were generous gifts of the Boehringer Co. Limit dextrin was prepared and purified as described earlier (Klein et al., 1982). Glucose-1,2-P was prepared by cyclization of glucose-1-P with carbodiimide in aqueous pyridine according to Zmudzka & Shugar (1964). Heptenitol was synthesized according to Hehre et al. (1980). [U-^{14}C]glucose-1-P (279 mCi/mM) and [^{32}P]phosphoric acid (200 mCi/mM) were from Amersham, England. All biochemicals and chemicals used were of the highest grade commercially available.

PP-α-glucose was synthesized from benzyl phosphate and glucose-1-P by using [U-^{14}C]glucose-1-P as tracer and the anion-exchange method of Michelson (1964). The corresponding α-D-glucose 1-(benzyl diphosphate) was hydrogenated to PP-α-glucose in 50 mM acetic acid sodium acetate, pH 4.8, with catalytic amounts of palladium charcoal (10%). Crude PP-α-glucose was subjected to chromatography on a column (1 × 18 cm) of silica gel 60 with a propanol/water gradient up to 1:1, yielding pure PP-α-glucose with R_f 0.43 on silica gel 60 thin-layer plates with solvent system A. The ^{31}P NMR spectrum is shown in Figure 1A.

PP-α-[U-^{14}C]glucose was synthesized from benzyl phosphate and [U-^{14}C]glucose-1-P according to the above procedure yielding PP-α-[U-^{14}C]glucose with a specific activity of 0.5 μCi/μmol.

Heptulose-2-P was formed by reaction of 150 mM heptenitol with 2 × 10^{-4} M potato phosphorylase in the presence of 150 mM [^{32}P]P_i (3 μCi/mmol) in 2 mM Na_2EDTA/10 mM Mops buffer, pH 6.8, at 30 °C for 24 h. The total volume was 2.5 mL. Incorporation of radioactivity into the product was followed by thin-layer chromatography with 2-propanol/NH_3/water, 7/2/1, as solvent and radioscanning. The phosphate ester was recovered by DE-52 (Whatman) ion-exchange chromatography by applying a linear ammonium

[2] Abbreviations: heptenitol, 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol; heptulose-2-P, 1-deoxy-D-*gluco*-heptulose 2-phosphate; PP-α-glucose, α-D-glucopyranose 1-diphosphate; glucose-1,2-P, α-D-glucopyranose cyclic 1,2-phosphate; D-glucal, 1,5-anhydro-2-deoxy-*arabino*-hex-1-enitol; glucose-1-P, α-D-glucopyranose 1-phosphate; pyridoxal-PP-α-glucose, pyridoxal(5′)diphospho(1)-α-D-glucose; α-glucan phosphorylase, 1,4-α-D-glucan:orthophosphate α-glucosyltransferase (EC 2.4.1.1); pyridoxal-P, pyridoxal 5′-phosphate; pyridoxal-PP, pyridoxal 5′-diphosphate; P_i, inorganic (ortho)phosphate; As, (ortho)arsenate; PP_i, pyrophosphate; AMP-S, adenosine 5′-(thiomonophosphate); AMP, adenosine 5′-monophosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, NMR, nuclear magnetic resonance; ppm, parts per million.

bicarbonate gradient (0–70 mM), pH 8.0. The lyophilized product was stored at −20 °C. A ^{31}P NMR spectrum of heptulose-2-P is shown in Figure 4C. For further identification, heptulose-2-P was hydrolyzed in 15 mM acetic acid at 60 °C for 20 min and cochromatographed on thin-layer plates with identical 1-deoxy-D-*gluco*-heptulose (Hehre et al., 1980).

Coenzymes. Pyridoxal-P was from E. Merck, Darmstadt, West Germany Pyridoxal 5′-phosphate monomethyl ester, prepared according to Pfeuffer et al. (1972), was a generous gift of Dr. Klaus Schnackerz of this laboratory. Pyridoxal 5′-(fluorophosphate) was prepared from pyridoxal-P as described by Klein et al. (1982). 5′-Deoxypyridoxal-5′-(methylenephosphonate) was kindly donated by Dr. O. Saiko (Merck, Darmstadt, West Germany).

Enzymes. Phosphorylase *b* was prepared from frozen rabbit skeletal muscle as described by Fischer & Krebs (1958). The enzyme was at least 3 times recrystallized and stored at 4 °C under toluene vapor. Muscle apophosphorylase *b* was prepared from phosphorylase *b* and reconstituted with pyridoxal-P and derivatives as described elsewhere [Hedrick et al., 1966; Vidgoff et al., 1974; Feldmann et al., 1976; cf. Klein et al. (1982)]. Potato phosphorylase was prepared from potato tubers according to Staerk & Schlenk (1967). The purified enzymes were homogeneous in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Primer was removed by treating phosphorylases repeatedly with Sepharose-bound α-glucoamylase according to Schiefer et al. (1978).

Analytical Procedures. Thin-layer chromatography was performed on silica gel 60 plates from Merck. Paper chromatograms were run in ascending mode on Schleicher & Schüll paper 2043. Solvent systems used were (A) ethanol/NH_3/water, 6:3:3, and (B) methanol/NH_3/water, 7:2:1. Sugars were visualized on silica gel plates with H_2SO_4 and heated for 10 min at 100 °C. Radioactive samples on thin-layer plates or paper strips were traced with a thin-layer scanner II of the Berthold Co., West Germany. Radioactivity was measured in a Triton X-100 liquid scintillation cocktail in a Packard Tri-Carb Model 3380. Protein concentrations were determined spectrophotometrically by using the extinction coefficients $A_{280}^{1\%} = 13.2$ for rabbit skeletal muscle phosphorylase (Kastenschmidt et al., 1968) and $A_{280}^{1\%} = 11.7$ for potato phosphorylase (Kamogawa et al., 1968). Enzyme concentrations are expressed in terms of monomer molecular weights of 97412 for muscle phosphorylase *a* (Titani et al., 1977) and 108000 for potato phosphorylase (Iwata & Fukui, 1973). When protein concentrations were determined with the Lowry method (Lowry et al., 1951), bovine serum albumin was used as a standard.

Activity Measurements. Activity assays were carried out routinely in the direction of glycogen synthesis. With rabbit muscle phosphorylase *b*, 16 mM glucose-1-P, 1% glycogen, and 1 mM AMP in 25 mM potassium maleate, 25 mM 2-mercaptoethanol, and 2 mM Na_2 EDTA buffer, pH 6.8, at 30 °C were used, and with potato phosphorylase, 10 mM glucose-1-P and 10 mM maltoheptaose were used. P_i was measured by the method of Fiske & Subbarow (1925).

NMR Measurements. Fourier-transformed ^{31}P NMR spectra were measured at 72.86 MHz in a Bruker WH-180 spectrometer as described in detail by Klein et al. (1982).

Results

Reaction of Pyridoxal Phosphorylase with PP-α-glucose. Formation of pyrophosphate from PP-α-glucose was followed by ^{31}P NMR spectroscopy (Figure 1) and incorporation of the glucosyl moiety by measuring the ^{14}C radioactivity in saccharides (Figure 2). The resonances in the ^{31}P NMR spectrum of PP-α-glucose bound to pyridoxal phosphorylase *b* at −11.5 ppm and at −6.2 ppm were assigned to the α- and β-phosphates of PP-α-glucose (Figure 1A). Assignments were made by comparison with the corresponding proton-coupled spectra of phosphorylase bound (not shown) and of free PP-α-glucose (Figure 1A, insert). PP-α-glucose did not measurably react with nonactivated pyridoxal phosphorylase without AMP in 12 h of incubation at room temperature (see control, Figure 2). Addition of limit dextrin and activation of the pyridoxal phosphorylase derivative with AMP-S caused drastic changes in the ^{31}P NMR spectrum (Figure 1B). We have substituted AMP-S for AMP to avoid interference with ^{31}P NMR resonances of P_i or glucose-1-P [cf. Feldmann & Hull (1977)]. AMP-S is even a better activator of native muscle phosphorylase *b* than AMP according to Murray & Atkinson (1968). Instead of the two separate peaks of PP-α-glucose resonances as in Figure 1A, a singlet appeared at −7.2 ppm, which was assigned to pyrophosphate by comparison with authentic pyrophosphate. The symmetry in the pyrophosphate peak formed is in accordance with a cleavage at the glucose ester position. This assumption was proved by thin-layer chromatography of the sample from the NMR experiment after splitting of the original pyrophosphate ester (not shown). From Figure 2, we have calculated an apparent $t_{1/2}$ of decomposition of the enzyme–substrate complex from incorporation of radioactive glucose into limit dextrin of ~4 min under these experimental conditions. This may be compared with a $t_{1/2}$ of ~13 min in the experiments reported by Takagi et al. (1982) with pyridoxal-PP-α-glucose-reconstituted muscle phosphorylase *b*. The mode of binding of the radioactive glucosyl residues incorporated into limit dextrin was determined by arsenolysis with AMP-activated phosphorylase *b*. The radioactivity was nearly quantitatively recovered as free glucose (see Figure 3B). Thus, the known specificity of phosphorylase-catalyzed arsenolysis (phosphorolysis) of α-(1→4) glycosidic bonds (Brown & Cori, 1961) allows the conclusion that the glucosyl residues arising from PP-α-[U-^{14}C]glucose and transferred to limit dextrin were linked by α(1→4) glycosidic bonds to the acceptor.

Table I: Kinetic Constants for Glucose-1-P, Glucose-1,2-P, and Heptulose-2-P[a]

enzymes	K_m for glucose-1-P (μM)	K_i for glucose-1,2-P (μM)	K_i for heptulose-2-P (μM)
muscle phosphorylase *b*	3.6 ± 0.6	500[b]	14 ± 1.0
potato phosphorylase	1.4 ± 0.3	70[c]	1.9 ± 0.2

[a] The reactions were carried out at 30 °C with 1×10^{-7} M rabbit skeletal muscle phosphorylase *b*, 1% limit dextrin, 1 mM AMP, and up to 20 mM glucose-1-P in 2 mM Na_2EDTA/50 mM 2-mercaptoethanol/50 mM Mops buffer, pH 6.8. Potato phosphorylase concentrations were 5×10^{-6} M, limit dextrin was replaced with 10 mM maltoheptaose, and AMP was omitted. The apparent K_m values were estimated from double-reciprocal plots of initial velocities of the phosphorylase reactions without and with concentrations of heptulose-2-P up to 25 μM. Inhibition by heptulose-2-P was strictly competitive at all concentrations of glucose-1-P; K_i values were calculated in the usual manner. All values represent determinations in triplicate. [b] Data from Hu & Gold (1978). [c] Data from Kokesh et al. (1977).

Heptulose-2-P as ^{31}P NMR Probe for Phosphate–Phosphate Interactions in Phosphorylase. Heptulose-2-P binds to potato phosphorylase and to muscle phosphorylase *b* about 40 times more strongly than does glucose-1,2-P, another competitive inhibitor, with respect to glucose 1-phosphate and phosphorylase (Kokesh et al., 1977; Hu & Gold, 1978) (Table I). Heptulose-2-P unlike glucose-1,2-P is protonatable around neutral pH. The pK_{II} of its phosphate was determined to be

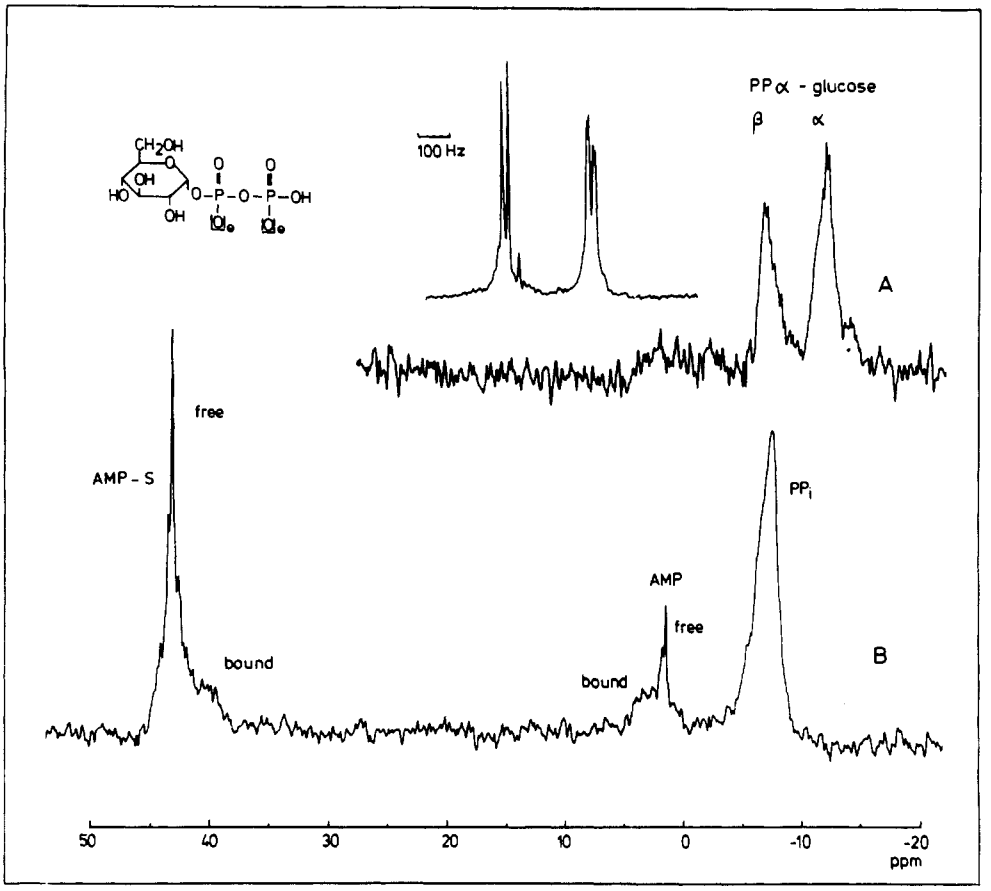


FIGURE 1: ^{31}P NMR spectra of reaction of pyridoxal phosphorylase *b* with PP-α-glucose. (A) Nonactivated rabbit muscle phosphorylase *b* reconstituted with pyridoxal, 3.9×10^{-4} M, in 2 mM Na_2EDTA, 50 mM 2-mercaptoethanol, 100 mM KCl, and 100 mM Mops buffer, pH 6.5, and 1.0 mM PP-α-glucose (27 000 transients). Inserted is the ^1H-coupled ^{31}P NMR spectrum of free PP-α-glucose in above buffer: $J_{P,P}$ = 20 Hz; $J_{H,P}$ = 5 Hz. (B) As in (A), but 1% limit dextrin and 1.2 mM AMP-S were also added (37 200 transients). All spectra were recorded at 24 ± 1 °C.

~6.7 by ^{31}P NMR titration (data not shown). This value is comparable to that for glucose-1-P (pK_{II} = 6.1). From model-building studies, it is apparent that the phosphate moiety of heptulose-2-P, in contrast to the phosphate ester of glucose-1-P, is drawn toward the neighboring hydroxy group (at C-3) of the pyranose ring to avoid interaction with the equatorial methyl group in the C-1 position. Under conditions identical with those of Figure 4C, the ^{31}P NMR signal of glucose-1-P is at 2.1 ppm. We assume that the 3 ppm upfield shift of the phosphate moiety of heptulose-2-P compared to glucose-1-P reflects the different geometric positions of the phosphates.

Thus, the ^{31}P resonance of heptulose-2-P is 2–6 ppm shifted upfield compared with phosphorylase-bound pyridoxal-P. In Figure 4A, the ^{31}P NMR spectrum of potato phosphorylase with heptenitol is shown. The resonance of the 5′-phosphate group of pyridoxal-P is at 4.5 ppm ($\Delta\nu$ = 50 Hz) and nearly identical with the resonance of pyridoxal-P without heptenitol (not shown). On addition of P_i, heptenitol is quantitatively converted to heptulose-2-P (Klein et al., 1984). The formation of heptulose-2-P significantly alters the resonance of the 5′-phosphate of the cofactor (Figure 4B): the ^{31}P resonance is shifted upfield from 4.5 to 3 ppm with considerable line broadening from $\Delta\nu$ = 50 to $\Delta\nu \geq 150$ Hz. The resonance line of the phosphate group of bound heptulose-2-P is concomitantly shifted downfield from −1.1 ppm for free heptulose-2-P to 0.2 ppm. The sharp singlet at 2.15 ppm ($\Delta\nu$ = 5 Hz) represents the ^{31}P resonance of P_i. The ^{31}P NMR resonances of the free compounds are given for comparison in Figure 4C. The chemical shift and line broadening of the 5′-phosphate group of pyridoxal-P and of the phosphate group of heptulose-2-P point to an interaction between both phosphates bound to potato phosphorylase. The chemical shift and line-width broadening could reflect exchange broadening due to a protonation–deprotonation equilibrium. The changes in the resonance of the 5′-phosphate group of the cofactor in the presence of heptulose-2-P are similar to the changes of the 5′-phosphate resonance of potato phosphorylase on addition of arsenate and glucose. The latter data have already been reported by Klein & Helmreich (1979) and Helmreich & Klein (1980).

Ionization of Phosphorylase-Bound 5′-Deoxypyridoxal-5′-(methylenephosphonate). The ^{31}P NMR spectra of AMP-S-activated and nonactivated rabbit muscle phosphorylase *b* reconstituted with 5′-deoxypyridoxal-5′-(methylenephosphonate) are shown in Figure 5. If we use the nomenclature of Feldmann & Hull (1977) for the natural pyridoxal phosphate containing rabbit muscle phosphorylases, the resonance at 14.4 ppm in Figure 5A for the nonactivated 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase *b* derivative should be designated as form I and the resonance at 22.0 ppm of the AMP-S-activated phosphorylase derivative as form III (Figure 5C). For the assignment of the ionization states of form I and form III of 5′-deoxypyridoxal-5′-(methylenephosphonate), one must take into account that the pH-dependent ^{31}P NMR chemical shifts on titration of the phosphonate in a model Schiff base formed from 5′-deoxypyridoxal-5′-(methylenephosphonate) and *N*-acetyllysine methyl ester are inverse of the shifts for the phosphate group of the corresponding Schiff base with pyridoxal-P (Schnackerz

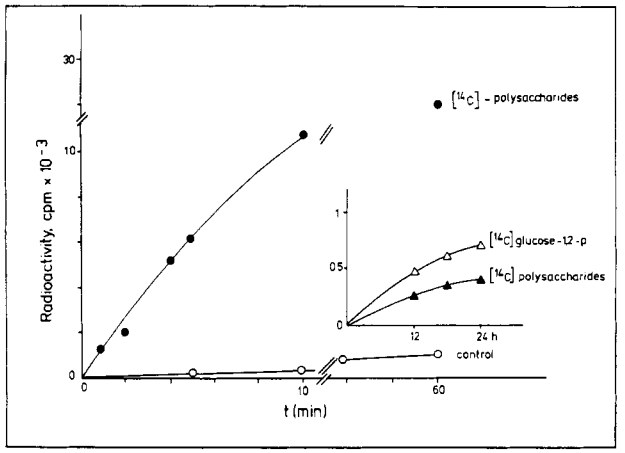


FIGURE 2: Fate of PP-α-glucose in reactions with pyridoxal-reconstituted muscle phosphorylase *b*. The reactions were carried out at 30 °C with 3.5×10^{-4} M PP-α-[^{14}C]glucose (1000 cpm/nmol), 1×10^{-4} M pyridoxal phosphorylase *b*, 1 mM AMP, and 1% limit dextrin where indicated. The buffer was 50 mM Mops, 50 mM 2-mercaptoethanol, and 2 mM Na_2EDTA, pH 6.5. The final volume was 1 mL. At the times given, 100-μL aliquots were withdrawn, and the reaction was quenched with 50 μL of MeOH. Incorporation of radioactive glucose into limit dextrin was measured with the filter paper assay of Thomas et al. (1958). Alternatively, polysaccharides and sugar phosphates were separated after completion of the reaction by paper chromatography in solvent system A, and radioactivity was measured (see Experimental Procedures). (●) Incorporation of [^{14}C]glucose in limit dextrin by pyridoxal phosphorylase *b*, activated with 1 mM AMP. (○) Control; incorporation of [^{14}C]glucose in limit dextrin in the presence of pyridoxal phosphorylase *b* without added AMP. (Insert) Reactions of AMP-activated pyridoxal phosphorylase *b* with PP-α-[^{14}C]glucose but without limit dextrin: (△) formation of [^{14}C]glucose-1,2-P; (▲) formation of ^{14}C-labeled polysaccharides. Note the expanded scale on the ordinate.

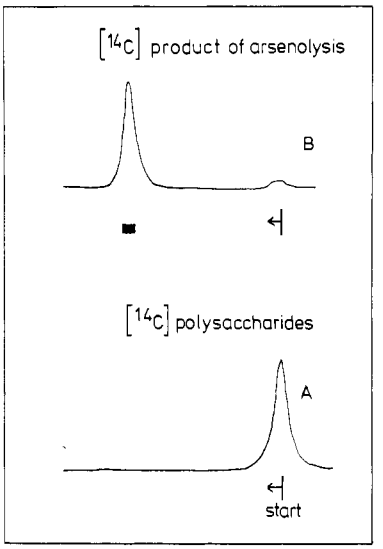


FIGURE 3: Radioactivity scans of arsenolysis of ^{14}C-labeled saccharides after glucosyl transfer from PP-α-[^{14}C]glucose. A sample (400 μL) of ^{14}C-labeled saccharides obtained as described in the legend to Figure 2 was passed over a DEAE-Sephadex column (0.4 × 4 cm) in the acetate form, and the retained material was eluted with H_2O (3 mL). The solution was concentrated in vacuo to ~500 μL and added to a solution containing 1×10^{-5} M rabbit muscle phosphorylase *b*, 1 mM 5′-AMP, and 20 mM As in 2 mM Na_2EDTA, 25 mM 2-mercaptoethanol, and 25 mM Mops buffer, pH 6.8. The final volume was 1 mL. The reaction mixture was incubated for 24 h at 25 °C, chromatographed on a DEAE-Sephadex column (0.4 × 4 cm), acetate form, eluted with H_2O, and concentrated to ~500 μL. The radioactivity scans are from silica gel 60 thin-layer chromatograms developed in solvent B: (A) ^{14}C-labeled saccharides; (B) product of arsenolysis. The box (■) indicates a sample of cochromatographed glucose.

& Feldmann, 1980). On the basis of this titration, the ^{31}P resonances at 14.4 and 22.0 ppm of the 5′-(methylenephosphonate) group of the cofactor analogue bound to phosphorylase *b* in the nonactivated and AMP-S-activated state represent dianionic and monoprotonated ionization states, respectively. The striking inversion of ionization may be a consequence of different geometries of the cofactor–enzyme and the cofactor analogue–enzyme complexes. Thus, the P–C bond is slightly longer (1.79 Å) than the P–O bond (1.61 Å) (Yount, 1975). Furthermore, one might anticipate that a CCP bond angle is smaller than a COP bond angle. This assignment is supported by the ^{31}P NMR spectra of the 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase *b* derivative in the presence of glucose-1,2-P: when glucose-1,2-P is added to the nonactivated 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase *b* derivative, the ^{31}P resonance of the dianionic 5′-(methylenephosphonate) group at 14.4 ppm is broadened beyond recognition (Figure 5B) or overlaps with the ^{31}P resonance line of glucose-1,2-P at 11.3 ppm. Either of these possibilities points to a more rigid dianionic 5′-(methylenephosphonate) group. On the other hand, in the case of the AMP-S-activated phosphorylase derivative with the monoprotonated 5′-(methylenephosphonate) group, the ^{31}P NMR resonance line was only slightly broadened on addition of the cyclic sugar phosphate (Figure 5D). The tightening of the binding of AMP-S to the activated 5′-deoxypyridoxal-5′-(methylenephosphonate)–phosphorylase on addition of glucose-1,2-P proved that the sugar phosphate was bound to the enzyme under these conditions (see Figure 5D). These experiments indicate that the monoanion of glucose-1,2-P (pK_a = 2.1), which is not protonatable in the pH range where phosphorylase is active, interacts much more strongly with the dianionic than with the monoprotonated form of the 5′-(methylenephosphonate) group of the cofactor. This conclusion is supported by experiments with AMP-S activated 5′-deoxypyridoxal-5′-(methylenephosphonate)–phosphorylase *b* and heptulose-2-P (Figure 5E): the ^{31}P resonance line of 5′-(methylenephosphonate) is centered at 15.7 ppm, and the resonances of free and bound heptulose-2-P are at −1.1 ppm. The small sharp peak at 2.3 ppm is probably due to P_i (Figure 5E). Comparing this experiment with the corresponding experiment with active native potato phosphorylase and heptulose-2-P (see Figure 4B), one arrives at the conclusion that the upfield shift of the 5′-(methylenephosphonate) resonance is induced by interaction of the mobile monoprotonated cofactor analogue and heptulose-2-P. Hence, in active 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase *b* the mobile monoprotonated 5′-(methylenephosphonate) group shares a proton with the phosphate group of heptulose-2-P. We conclude that this interaction is relevant for catalysis because it occurs only with the activated enzyme and moreover because heptulose-2-P, unlike glucose-1,2-P, is protonatable in the pH range where the enzyme is active. In active potato phosphorylase the sequence of protonation of the cofactor phosphate is reversed. The natural cofactor, pyridoxal-P, is dianionic and is protonated on interaction with heptulose-2-P. The implications of these observations for the role of the phosphorus of the 5′-phosphate side chain of pyridoxal-P as proton donor in phosphorylase catalysis are evident. They will be discussed later.

Activation of Pyridoxal Phosphorylase by Anions. Table II demonstrates that noncovalently bound anions, regardless of whether they are protonatable or not in the pH range of phosphorylase catalysis, are capable of significantly reactivating pyridoxal-containing rabbit muscle phosphorylase *b*. This agrees with the data reported originally by Parrish et al.

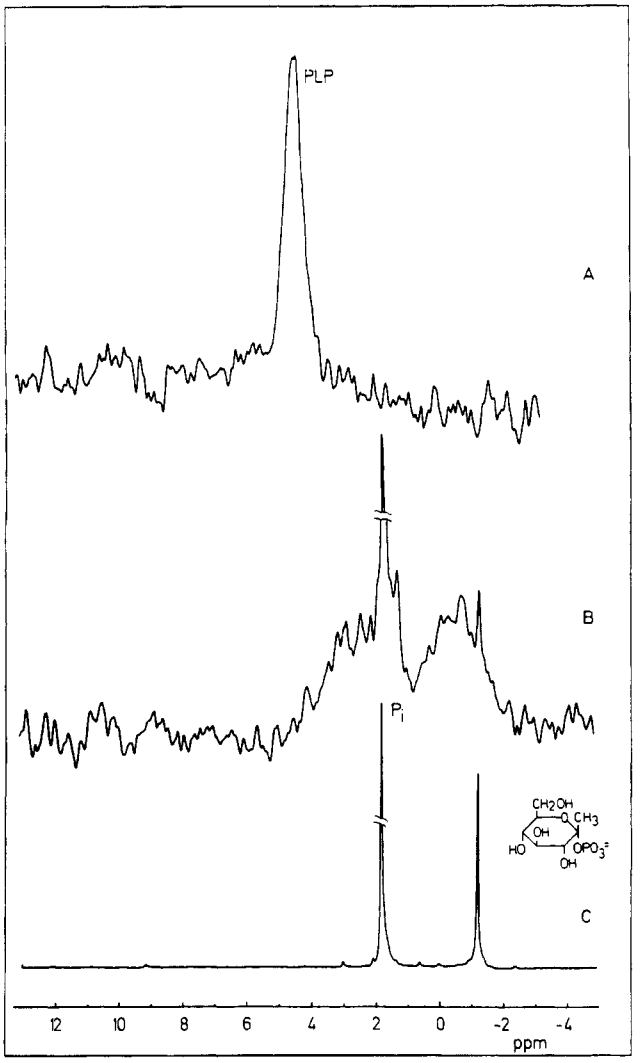


FIGURE 4: ^{31}P NMR spectra of potato phosphorylase in the presence of heptenitol. The proton-decoupled spectra were recorded at 24 ± 1 °C: (A) 4×10^{-4} M potato phosphorylase and 1.0 mM heptenitol in 2 mM Na_2EDTA/25 mM Mops buffer, pH 6.9 (total volume was 11 mL; 20642 transients); (B) like (A), but after 2 h reaction with 1.2 mM P_i (51000 transients); (C) reference containing no enzyme but 2 mM P_i and 2.5 mM heptulose-2-P in 2 mM Na_2EDTA/25 mM Mops buffer, pH 6.9 (430 transients).

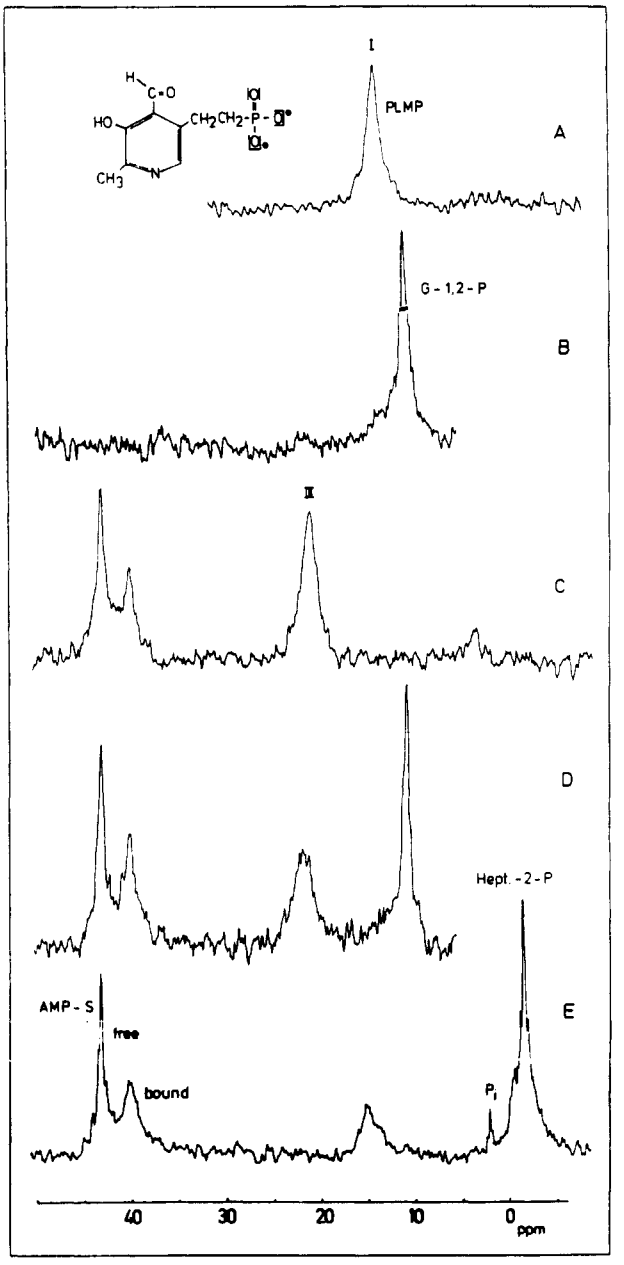


FIGURE 5: ^{31}P NMR spectra of 5′-deoxypyridoxal-5′-(methylenephosphonate) (PLMP) reconstituted rabbit muscle phosphorylase *b*. All reactions were carried out with 4×10^{-4} M phosphorylase derivative in 2 mM Na_2EDTA, 50 mM 2-mercaptoethanol, 100 mM KCl, and 100 mM Mops buffer, pH 7.0, at 24 ± 1 °C: (A) no AMP-S (33500 transients); (B) no AMP-S but with 1.4 mM glucose-1,2-P (G-1,2-P) (36500 transients); (C) enzyme activated with 0.9 mM AMP-S (27000 transients); (D) enzyme activated with 0.9 mM AMP-S in the presence of 1.2 mM glucose-1,2-P and 0.5% limit dextrin (20500 transients); (E) enzyme activated with 1.05 mM AMP-S, 2 h after reaction in the presence of 0.5% limit dextrin, 4.5 mM heptenitol, and 1.25 mM P_i (43200 transients). Hept-2-P is heptulose-2-P.

(1977) and subsequently by Withers et al. (1982a) and Chang et al. (1983). However, these anions bound covalently to the 5′-CH_2OH side chain of pyridoxal form pyridoxal 5′-phosphate analogues that activate rabbit muscle phosphorylase *b* only when they are protonatable in the pH range where phosphorylase is active (Pfeuffer et al., 1972). The implications of this fact for the assumed role of the 5′-phosphate group of the natural cofactor pyridoxal-P as an acid in catalysis by α-glucan phosphorylases will now be discussed.

Discussion

Reaction of Pyridoxal-Reconstituted Muscle Phosphorylase with PP-α-glucose. Pyridoxal phosphorylase requires a phosphate, phosphite, or fluorophosphate dianion as activator to catalyze glucosyl transfer from glucose-1-P, its natural substrate, to an acceptor. The "activating" phosphate was thought to substitute for the 5′-phosphate of pyridoxal-P in the native enzyme. In PP-α-glucose, the activating phosphate is linked via a pyrophosphate bond to the phosphate of glucose-1-P. We have shown that PP-α-glucose serves as substrate for pyridoxal-reconstituted muscle phosphorylase *b*. On the basis of the data reported by Takagi et al. (1982), the rate of glucosyl transfer from PP-α-glucose seems to be even faster than the corresponding rate from PP-α-glucose linked covalently to pyridoxal. Moreover, glucosyl transfer from PP-α-glucose to limit dextrin, like the corresponding glucosyl transfer from pyridoxal-PP-α-glucose, is stereospecific, forming α-(1→4) glycosidic bonds. The demonstrated reactions of pyridoxal-PP-α-glucose as a transition-state analogue were considered to support a mechanism involving electrophilic catalysis by the 5′-phosphate of the natural cofactor pyridoxal-P (Withers et al., 1982b). The assumption that a pseudo-pyrophosphate bond is formed in phosphorylase catalysis is analogous to an abortive phospho-transfer mechanism.

Table II: Reactivation of Muscle Phosphorylase by Phosphate and Phosphate Derivatives Bound Noncovalently or Bound Covalently to Pyridoxal[a]

muscle phosphorylase *b* prepn containing	activity (μmol min^{-1} mg^{-1})
pyridoxal phosphate	60
pyridoxal (methyl phosphate)	<1
pyridoxal fluorophosphate	<1
pyridoxal	<1
pyridoxal + PO_4H^{2-} (pK_{II} = 7.1)	4.9
pyridoxal + PO_4Me^{2-} (pK_{II} = 6.3)	5.3
pyridoxal + PO_3F^{2-} (pK_{II} = 4.7)	5.7

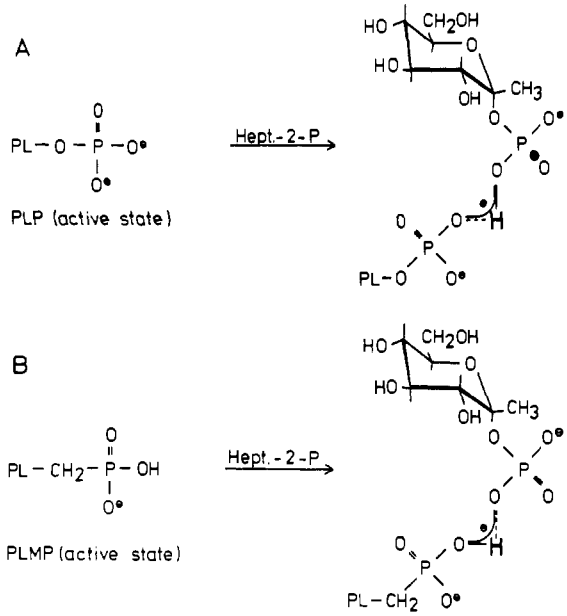
[a] All reactions were carried out in triplicate with 30 mM [U-^{14}C]-glucose-1-P (50 cpm/nmol), 1% limit dextrin, and 1 mM AMP in 25 mM 2-mercaptoethanol/1 mM Na_2EDTA buffer, pH 6.8, at 30 °C. The concentration of enzyme was 2×10^{-6} M except for native pyridoxal-P containing rabbit muscle phosphorylase *b* where the concentration was 1×10^{-7} M. The concentrations of anions were 7.5 mM. Rates in direction of glycogen synthesis were calculated from the linear part of the progress curves. Incorporation of radioactive glucose into limit dextrin was measured with the filter paper assay of Thomas et al. (1968) (see Experimental Procedures).

This would require according to Withers et al. (1982a) "tight coordination of the pyridoxal-phosphate oxygens not only to facilitate a trigonal-bipyramidal configuration of the phosphorus but also to prevent the splitting off of pyridoxal (or water) by an in-line attack of the substrate phosphate". Another possibility of stabilizing the 5′ oxygen–phosphate bond would be its equatorial positioning in a trigonal-bipyramidal transition state [cf. Knowles (1982)]. But be that as it may, the reaction of PP-α-glucose with pyridoxal phosphorylase described above clearly indicates that for the activation of the glucosyl moiety in pyridoxal-PP-α-glucose (Withers et al., 1981b; Takagi et al., 1982), a covalent bond with the 5′ side chain of the cofactor is not necessary. The similar rates of glucosyl transfer show that pyrophosphate and pyridoxal-PP are comparable leaving groups. Thus, the reaction of pyridoxal phosphorylase with PP-α-glucose merely points to the requirement for an activating phosphate in addition to the requirement for a substrate phosphate group. But neither the reaction of pyridoxal-PP-α-glucose nor the reaction of PP-α-glucose can be expected to give information as to the transition state in phosphorylase catalysis.

Reactivation of Pyridoxal Phosphorylase by Anions. The main objection to a role for the phosphorus of pyridoxal-P as an acid in phosphorylase catalysid (Klein & Helmreich, 1979) comes from Parrish's et al. (1977) observation that fluorophosphate (pK_{II} = 4.8) is as effective as/or even more effective than phosphite (pK_{II} = 6.6) or phosphate (pK_{II} = 7.1) in partially reactivating pyridoxal-reconstituted phosphorylase [Withers et al., 1982a; see also Chang et al. (1983)]. The similarity of the pH profiles of pyridoxal phosphorylases reactivated with either fluorophosphate or phosphite was taken to support this objection (Withers et al., 1982c). Incidentally, the pH activity optima for the 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase derivative and the native pyridoxal-P-containing enzyme are also similar. In the case of the phosphorylase derivative, the pH optimum for activity is shifted only by 0.2 pH unit to the basic side compared with the native enzyme, whereas the 5′-phosphonate-containing cofactor compared with pyridoxal 5′-phosphate has a pK_{II} value a whole pH unit more basic (Vidgoff et al., 1974). All these arguments rest on the tacit assumption that orthophosphate and pyridoxal bind to the same sites in pyridoxal phosphorylase to which pyridoxal-P is bound in the native enzyme. But, this assumption neglects the differences in the distance between the phosphorus and the 5′-oxygen when the phosphorus is bound covalently to pyridoxal or not. In the latter case, the distance is at least 2.8 Å greater. These differences between a noncovalently bound phosphate or other dianion as compared to the same anion immobilized by a covalent bond with the 5′ side chain of pyridoxal would seem to make it unlikely that noncovalently bound anions—whatever their p*K*—can replace the 5′-phosphate of the cofactor as proton donor–acceptor group (Klein & Helmreich, 1979; Klein et al., 1982). Perhaps, a slightly different structure of the pyridoxal phosphorylase–anion complex, compared to the native holoenzyme, might bring in a proton donor group of the enzyme that normally is not in the reaction path. Actually, the similar pH profiles of the anion-activated pyridoxal phosphorylases mentioned above point to an amino acid side chain of the enzyme rather than the activating anion as determinant of the pH activity relations (Withers et al., 1982c). Thus, in reactions of anion-activated pyridoxal phosphorylase (including the reaction with PP-α-glucose), an amino acid side chain of the enzyme might bypass the noncovalently bound anions regardless of whether they are protonatable or not. The assignment of an amino acid side chain as proton donor in pyridoxal phosphorylase, however, is a matter of speculation. On the basis of the structure of phosphorylase *a* at 2.1-Å resolution (Sprang et al., 1982), one might consider Asp-283. Another candidate is His-570, which is near the phosphate of glucose-1-P. This residue is however not well localized in the electron-density map of phosphorylase *b* at 2-Å resolution (Sansom et al., 1984). But be that as it may, the data in Table II stress that the dianionic group when immobilized by a covalent bond with the 5′-CH_2OH group of pyridoxal must be protonatable in the pH range where phosphorylase is active, in order for the enzyme to function catalytically.

Role of the 5′-Phosphate Group of Pyridoxal-P in Catalysis. Mainly on the basis of the reaction of α-glucan phosphorylases with D-glucal, we have suggested that the phosphate of the substrates and the 5′-phosphate of pyridoxal-P participate in a general acid catalysis (Klein et al., 1982). This proposal implies proximity of the phosphate groups, which is supported by the data of Withers et al. (1981) and by the data obtained with heptulose-2-P as well (see Figures 4B and 5E). Heptulose-2-P effectively replaces glucose-1,2-P as an inhibitor specific for the glucose-1-P site. According to the 3.0-Å difference Fourier map, heptulose-2-P binds to phosphorylase *b* very much like glucose-1,2-P. In the heptulose-2-P–phosphorylase *b* complex, the phosphate oxygens are within hydrogen-bonding distance (2.7 Å) of the pyridoxal-P oxygens (McLaughlin et al., 1984). The close proximity of the phosphates is also apparent in the ^{31}P NMR spectrum of potato phosphorylase and heptulose-2-P (Figure 4B). In order to obtain a more refined picture, ^{31}P NMR spectra were also recorded with 5′-deoxypyridoxal-5′-(methylenephosphonate)-reconstituted muscle phosphorylase *b* [cf. Hörl et al. (1979)] (see Figure 5). In the nonactivated enzyme derivative the 5′-phosphonate is dianionic, whereas the 5′-phosphate of pyridoxal-P in the corresponding nonactivated phosphorylase *b* is monoprotonated (Feldmann & Hull, 1977). The inverted ionization has to be borne in mind for the comparison of the ^{31}P NMR spectra of the pyridoxal-5-P and the 5′-deoxypyridoxal-5′-(methylenephosphonate)-containing phosphorylases, particularly when glucose-1,2-P or heptulose-2-P is present (Figures 5D,E and 4B): in the activated muscle enzyme derivative the phosphonate group is monoprotonated due to its higher p*K* (7.4 vs. 6.3), but since glucose-1,2-P cannot accept a proton from the 5′-phosphonate

Scheme I: Phosphate–Phosphate Interaction[a]



[a] (A) Interaction of 5′-phosphate of pyridoxal-P in active potato phosphorylase with heptulose-2-P. (B) Interaction of 5′-phosphonate of 5′-deoxypyridoxal-5′-(methylenephosphonate) in rabbit muscle phosphorylase *b* with heptulose-2-P.

group, the NMR spectral change is minor, and accordingly, the line width is only slightly broader without chemical shift (Figure 5D). This should be contrasted to the changes of the dianionic forms: in the presence of glucose-1,2-P (Figure 5B), the dianionic phosphonate group of 5′-deoxypyridoxal-5′-(methylenephosphonate) becomes more rigid. But from that it does not follow that the corresponding tightening of the dianionic phosphate structure in the case of the natural cofactor on binding of glucose-1,2-P must be relevant for catalysis as was implied by Withers et al. (1981a). In this context, it needs to be emphasized that glucose cyclic 1,2-phosphate, unlike heptulose-2-P, cannot accept a proton. And indeed, when glucose cyclic 1,2-phosphate is replaced by heptulose-2-P, the monoprotonated 5′-phosphonate group of the active 5′-deoxypyridoxal-5′-(methylenephosphonate) phosphorylase derivative is partially deprotonated (Figure 5E). Correspondingly, the 5′-phosphate of the natural cofactor pyridoxal-P, which is dianionic in active potato phosphorylase, shares a proton with the phosphate of heptulose-2-P (Figure 4B). We therefore consider the experiments with heptulose-2-P to be more informative than experiments with glucose cyclic 1,2-phosphate: the ^{31}P NMR data with the protonatable inhibitory sugar phosphate with native pyridoxal-P phosphorylase and a partially active 5′-deoxypyridoxal-5′-(methylenephosphonate)–enzyme derivative clearly show ionization changes of the 5′-phosphate (-phosphonate) side chain of the cofactor. These findings can be interpreted plausibly by assigning the role of a proton–donor acceptor in phosphorylase catalysis to the 5′ side chain of pyridoxal-P (Scheme I). On the other hand, the experiments with heptulose-2-P virtually exclude a participation of the cofactor phosphorus as a constrained dianion (Withers et al., 1981a, 1982a).

The assumption that the phosphorus of the cofactor exists as a constrained dianion was based on ^{31}P NMR spectra of phosphorylases *b* and *a* with glucose-1,2-P (Withers et al., 1981a). But in the light of the experiments with heptulose-2-P, it now appears that this conclusion hinges on the nonprotonatability of the cyclic sugar phosphate in the pH range of the phosphorylase reaction. Thus, our original interpretation (Feldmann et al., 1978) gains credibility, although the path along which the proton travels remains to be charted. It is now for the X-ray crystallographers to test our hypothesis and fill the gaps still existing in our understanding of the role of the 5′-phosphate of pyridoxal-P in α-glucan phosphorylase catalysis. The presently attained level of atomic resolution should make that possible. Finally, we wish to raise the question what α-glucan phosphorylases may have gained by the involvement of the 5′-phosphate of pyridoxal-P as general acid catalyst. Sucrose phosphorylase, after all, manages to phosphorolyze glycosidic bonds without pyridoxal-P. Although the catalytic mechanisms, especially with respect to the intervention of a glucosyl intermediate, are probably very closely related, the two types of enzymes differ significantly: sucrose phosphorylase cannot handle oligo- or polysaccharides and is not primer dependent. Moreover, sucrose phosphorylase is much less restrictive with respect to glucosyl acceptors than are α-glucan phosphorylases. Thus, sucrose phosphorylase can transfer substrate glucosyl residues to other monosaccharides but also to phosphate or arsenate and, although 50 times less efficiently, even to water [cf. Silverstein et al. (1967)]. It is therefore tempting to speculate that, for sucrose phosphorylase, phosphate is an acceptor rather than a catalytic group involved in activation. In α-glucan phosphorylases on the other hand, interaction of the 5′-phosphate of the cofactor with the substrate phosphates increases, through an anchimeric effect, the acidity of the substrate phosphates, and the proton donor–acceptor function of the phosphate of pyridoxal-P enhances the nucleophilicity of orthophosphate. It is not difficult to imagine how inclusion of proton transfer might have selected for phosphorolysis and against hydrolysis by acquiring pyridoxal-P as the need arose to catalyze glucosyl transfer from hydrophilic oligo- or polysaccharides via an activated intermediate to orthophosphate without interference from water.

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Registry No. Pyridoxal-P, 54-47-7; pyridoxal-PP-α-glucose, 80202-75-1; PP-α-glucose, 92642-57-4; heptenitol, 74310-30-8; glucose-1-P, 59-56-3; heptulose-2-P, 92642-58-5; phosphorylase, 9035-74-9; phosphorylase *b*, 9012-69-5.

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