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Functions of the 5′-Phosphoryl Group of Pyridoxal 5′-Phosphate in Phosphorylase: A Study Using Pyridoxal-Reconstituted Enzyme as a Model System†

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ABSTRACT: Pyridoxal-reconstituted phosphorylase was used as a model system to study the possible functions of the 5′-phosphoryl group of pyridoxal 5′-phosphate (PLP) in rabbit muscle glycogen phosphorylase. Kinetic study was conducted by using competitive inhibitors of phosphite, an activator, and α-D-glucopyranose 1-phosphate (glucose-1-P) to study the relationship between the PLP phosphate and the binding of glucose-1-P to phosphorylase. Fluorine-19 nuclear magnetic resonance (^{19}F NMR) spectroscopy of fluorophosphate bound to pyridoxal phosphorylase showed that its ionization state did not change during enzymatic catalysis. Evaluation of the apparent kinetic parameters for the activation of pyridoxal phosphorylase with different analogues having varied pK_{a2} values demonstrated a dependency of K_M on pK_{a2}. Molybdate, capable of binding as chelates in a trigonal-bipyramidal configuration, was tested for its inhibitory property with pyridoxal phosphorylase. On the basis of the results in this study, several conclusions may be drawn: (1) The bound phosphite in pyridoxal phosphorylase and, possibly, the 5′-phosphoryl group of PLP in native phosphorylase do not effect the glucose-1-P binding. (2) One likely function of the 5′-phosphoryl group of PLP in native phosphorylase is acting as an anchoring point to hold the PLP molecule and/or various amino acid side chains in a proper orientation for effective catalysis. (3) The force between the PLP phosphate and its binding site in phosphorylase is mainly electrostatic; a change of ionization state during catalysis is unlikely. (4) Properties of the central atoms of different anions are important for their effects as either activators or inhibitors of pyridoxal phosphorylase. (5) Our results with molybdate are consistent but do not prove that a trigonal-bipyramidal structure of PLP is involved in the catalytic mechanism of phosphorylase.

Various studies of α-glucan phosphorylases reconstituted with vitamin B_6 analogues suggest that the 5′-phosphoryl group of PLP[1] is likely to be involved in the catalytic process [see reviews by Helmreich & Klein (1980) and Graves & Wang (1972)]. Although phosphorylase reconstituted with pyridoxal is inactive (Illingworth et al., 1958), it was found some phosphate analogues could activate the enzyme (Parrish et al., 1977). These findings were explained by binding of phosphate and analogues at the site where the 5′-phosphoryl group of the coenzyme in native phosphorylase resides. Inorganic pyrophosphate was found a potent inhibitor to pyridoxal-re-

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[1] Abbreviations: glucose-1-P, α-D-glucopyranose 1-phosphate; PLP, pyridoxal 5′-phosphate; NMR, nuclear magnetic resonance; ppm, parts per million; EDTA, ethylenediaminetetraacetic acid.

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constituted phosphorylase and was competitive with both the activator, phosphite, and the substrate, glucose-1-P. One mole of pyrophosphate bound per mole of pyridoxal phosphorylase. These findings suggested that the glucose-1-P binding site and the binding site of the 5′-phosphoryl group of PLP are in close proximity and the inhibition by pyrophosphate could occur by extending across the two sites (Parrish et al., 1977). The proximity between coenzyme and glucose-1-P has been firmly established by high-resolution X-ray crystallographic maps developed from two laboratories (Sygusch et al., 1977; Weber et al., 1978).

Several attractive mechanisms have been proposed for the involvement of the coenzyme phosphate in the catalytic mechanism. Johnson et al. (1980) suggested that the coenzyme phosphate may function as a dianion to carry out a nucleophilic attack on the C-1 carbon of glucose-1-P. From the studies of the effects of 1,2-dimethoxyethane on phosphorylase, coenzyme phosphate was suggested to be able to stabilize the glucosyl cation in the intermediate (Uhing et al., 1981). From extensive ^{31}P NMR studies, an alternative mechanisms was suggested in which the coenzyme dianionic phosphate acts as a proton acceptor–donor during the glucosyl-transfer reaction (Feldmann & Helmreich, 1976; Feldmann & Hull, 1977; Helmreich & Klein, 1980). Recent findings of Palm et al. (1983) with α-D-glucosyl fluoride and Klein et al. (1982) with α-D-glucal are consistent with this proposed mechanism. On the basis of studies with phosphorylase reconstituted with pyridoxal(5′)diphospho(1)-α-D-glucose, Withers et al. (1981) proposed an interesting mechanism in which the coenzyme phosphate could act as an electrophile, which would produce some inductive effect on the substrate and thereby labilize the glucosidic bond. In support of this mechanism, Takagi et al. (1982) showed that radioactive glucose from pyridoxal(5′)diphospho(1)-α-D-glucose could be incorporated into the nonreducing end of glycogen. The possibility that a direct interaction of the substrate and the coenzyme phosphate occurs is supported by the recent results of Withers et al. (1982) that showed the conformational state of phosphorylase reconstituted with pyridoxal pyrophosphate mimics the active state found in the native phosphorylase.

To gain a better understanding of the characteristics of the 5′-phosphoryl group of PLP in the native enzyme, further studies were done on phosphorylase reconstituted with pyridoxal. The use of this enzyme form allowed us to assess the influence of anions, varying in size, geometry, ionization state, and electronic configuration, bound to the phosphoryl site on catalysis and to evaluate the role of the coenzyme phosphoryl group in the binding of substrate, glucose-1-P.

Materials and Methods

Rabbit skeletal muscle was obtained from Pel-Freeze Co. [U-^{14}C]Glucose-1-P was purchased from Amersham. Methylphosphonic acid and ethylphosphonic acid were obtained from Alfa Products. (Aminomethyl)phosphonic acid and glucose-1-P were obtained from Sigma Chemical Co. Purified disodium fluorophosphate, a generous gift of Dr. S. C. Yan, was prepared following a modified method of Higgins & Baldwin (1955) as described by Yan & Graves (1982). Glucose cyclic 1,2-phosphate was synthesized from glucose-1-P according to the method of Zmudzka & Shugar (1964) modified by a procedure described by Dreyfus et al. (1980). All other materials were the highest quality commercially available.

Rabbit skeletal muscle glycogen phosphorylase was prepared according to Fischer & Krebs (1962) except that 30 mM 2-mercaptoethanol was substituted by cysteine. Apophosphorylase *b* was prepared by the method of Graves et al. (1975). No enzymatic activity could be detected from the apophosphorylase used in this study, even at high protein concentration (0.2 mg/mL). After incubation of apoenzyme (1 mg/mL) with a 10-fold excess of pyridoxal 5′-phosphate for 30 min at 30 °C, the resulting enzyme showed a specific activity of 40 μmol/(min·mg). Apophosphorylase *a* was prepared by the method of Uhing et al. (1981). Pyridoxal-reconstituted phosphorylase was prepared by the method of Parrish et al. (1977). Enzymatic activity in the direction of glycogen synthesis was measured either by liberation of inorganic phosphate from glucose-1-P described by Illingworth & Cori (1953) or by the incorporation of [^{14}C]glucose into glycogen with the filter paper assay of Thomas et al. (1968). When the activity was measured in the presence of anions, the conditions used are indicated in the appropriate figure legends. The protein concentration of phosphorylase was measured spectrophotometrically by using the extinction coefficient $E^{1\%}_{10mm}$ at 280 nm of 13.2, according to Kastenschmidt et al. (1968).

^{19}F NMR spectra were obtained at 282.4 MHz on a Bruker MW300 superconducting spectrometer operating at 20 °C. A spectral width of 10000 Hz was employed with a 20-μs pulse width and a repetition time of 1 s. Exponential line broadening used before Fourier transformation was 5 Hz. Ten percent D_2O (v/v) was present in the buffer used for field/frequency lock, and a 1-mm tube containing TFA (trifluoroacetic acid) was inserted for chemical shift referencing.

Results and Discussion

To determine the properties of anions necessary to activate pyridoxal phosphorylase, the effects of various anions on the enzymatic activity of the pyridoxal-reconstituted phosphorylase were studied. Because the effect of some of these ions had been reported earlier at one concentration (Parrish et al., 1977), studies were extended to evaluate effects at different concentrations (Figure 1), to evaluate kinetic parameters of activation, and to provide a basis for comparison for other ions not tested earlier. Pyridoxal phosphorylase *a* was used along with pyridoxal phosphorylase *b* because it is known that the phosphoryl group in the *a* form has a stabilizing effect on the pyridoxal 5′-phosphate binding site (Shaltiel et al., 1969). The results with pyridoxal phosphorylase *b* (Figure 1A) show that phosphate, phosphite, fluorophosphate, arsenate, sulfite, and selenite can reactivate the enzyme to different extents, whereas arsenite, sulfate, bicarbonate, nitrate, and nitrite from 0.1 to 5 mM showed no effect (not illustrated). Other anions, ClO_3^-, ClO_4^-, CrO_4^{2-}, $H_2BO_3^-$, F^-, and the divalent cations Mg^{2+} and Mn^{2+}, at 5 mM, had no effect. A similar effect of anions on pyridoxal phosphorylase *a* was found (Figure 1B) except it seems that less activation was afforded by selenite in this case. No activation of native phosphorylase *b* or *a* could be observed by these activators, showing that activation cannot be explained by any residual native enzyme enzyme in the pyridoxal enzyme preparations. In fact, sulfite, selenite, phosphate, and phosphite but not fluorophosphate at 5 mM caused some inhibition of native phosphorylase *b* (not illustrated). The extent of activation seen in Figure 1 is likely due to a balance of effects: (a) activation caused by binding at the coenzyme phosphoryl binding site and (b) inhibition by binding at the glucose-1-P site (Parrish et al., 1977). The apparent kinetic constants of the activators used in Figure 1 along with thiophosphate and some alkylphosphonates for pyridoxal-reconstituted phosphorylase *b* are shown in Table I.

In Table I, a great variation of K_m's (from 0.4 to 28.6 mM) but a comparably smaller variation of V_{max}'s (from 0.5 to 4.7

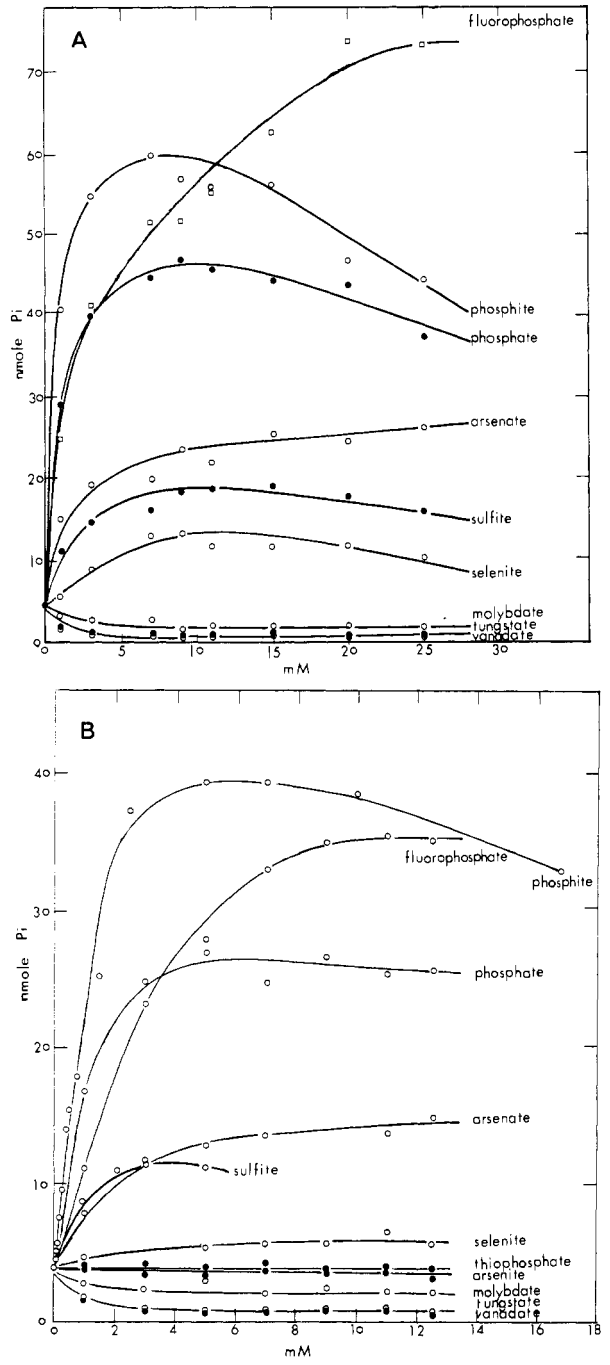


FIGURE 1: Effect of anions on enzymatic activity of pyridoxal-reconstituted phosphorylase. (A) The assay solutions at pH 6.8 and 30 °C contained pyridoxal-reconstituted phosphorylase *b* (78 μg/mL), 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.02 M β-glycerophosphate, 0.016 M [U-^{14}C]glucose-1-P, 1% glycogen, and anions at the indicated concentrations. At 5 min, 30 μL were removed, and the radioactivity incorporated into glycogen was measured as described under Materials and Methods. (B) Conditions were described in (A) except that no AMP was used and pyridoxal-reconstituted phosphorylase *b* was substituted by pyridoxal-reconstituted phosphorylase *a* (139 μg/mL).

IU) is seen among the different phosphate analogues. The distribution of K_m values seems to be dependent on the pK_{a2} values. Analogues with pK_{a2} higher than 7.1 have considerably higher K_m values. One exception of this generality is (aminomethyl)phosphonate, which has a low pK_{a2}, 5.9, and a high K_m value. The observed dependence of K_m value on pK_{a2} can be explained reasonably by assuming that the binding between phosphate analogues and protein is mainly through an electrostatic interaction; therefore, the analogues with higher pK_{a2}'s

Table I: Apparent Kinetic Parameters of Activator Anions on the Pyridoxal-Reconstituted Phosphorylase *b*[a]

anion	pK_{a2}	V_{max} [μmol/(min·mg)]	K_M (mM)
phosphite	6.6[b]	4.7	0.6
fluorophosphate	4.8[c]	3.9	1.1
phosphate	7.7	3.6	1.3
thiophosphate	6.2[c]	1.8	0.4
(aminomethyl)phosphonate	5.9[d]	2.0	12.1
ethylphosphonate	8.4[d]	2.6	28.6
methylphosphonate	7.9[d]	2.6	24.0
selenite	8.3[e]	1.8	3.1
sulfite	7.0[f]	1.1	1.1
thiosulfate	2.3[g]		0.8[h]
arsenate	6.9	0.5	1.9

[a] The reaction mixture at 30 °C and pH 6.8 contained 0.02 M β-glycerophosphate, 0.015 M 2-mercaptoethanol, 0.001 M AMP, 0.016 M glucose-1-P, 1% glycogen, varied concentrations of different anions (except for thiosulfate), and pyridoxal-reconstituted phosphorylase *b* (~200 μg/mL). [b] Weast (1974). [c] Van Wazer (1958). [d] Byers et al. (1979). [e] Kudryavtsev (1974). [f] Nickless (1968). [g] Yui & Hugisawa (1942). [h] This is the K_I value evaluated from the secondary plot of Figure 3B.

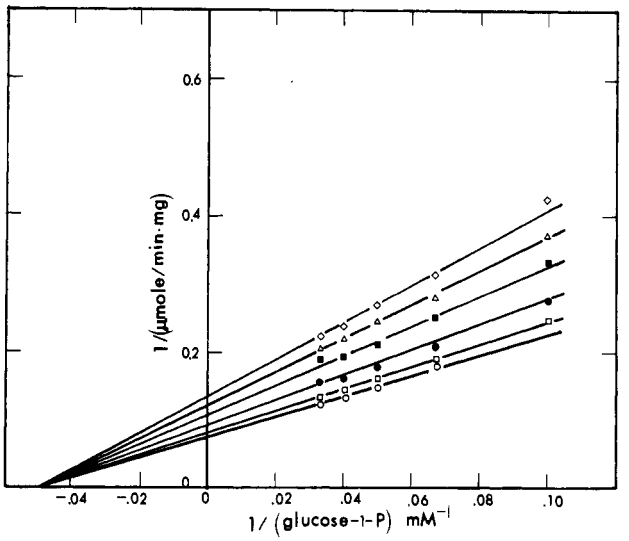
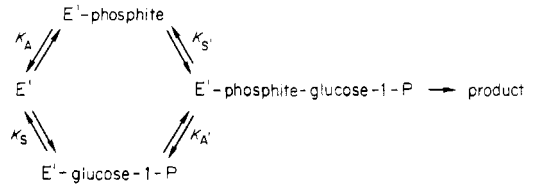


FIGURE 2: Double-reciprocal plot of initial reaction velocity *v* vs. [glucose-1-P]. Phosphite concentrations were held constant at 4.0 (○), 3.0 (□), 2.0 (●), 1.0 (■), 0.75 (△), and 0.5 mM (◇). The reaction mixtures at pH 6.8 and 30 °C consisted of pyridoxal-reconstituted phosphorylase *b* (155 μg/mL), 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, and 1% glycogen with glucose-1-P and phosphite at the indicated concentrations.

interact more weakly with protein because they carry less negative charge. Although (aminomethyl)phosphonate has a low pK_{a2} value, the partial positive charge carried by the amino group may lower the net negative charge of the whole molecule and weaken its binding to a positively charged site on the protein. Crystallographic studies of phosphorylase have shown that the 5′-phosphoryl group of PLP is surrounded by several basic amino acids. This also indicates that strong electrostatic interactions occur between this phosphoryl group and its surroundings in the protein.

Because the 5′-phosphoryl group of PLP resides closely to the glucose-1-P binding site on the enzyme, a possible function of this phosphoryl group is to provide a certain structural flexibility to the enzyme necessary for the binding of glucose-1-P. To test this possibility, kinetic studies were done to evaluate the binding of phosphite and glucose-1-P with pyridoxal-reconstituted phosphorylase *b*. Enzymatic activities were measured in the presence of variable phosphite and

Scheme I



glucose-1-P concentrations, and these data are shown in the Lineweaver–Burk plot of Figure 2. The arrangement of these lines in Figure 2, converging at the x axis, can be described by an equation derived for either a random or an ordered bireactant mechanism. Withers et al. (1982a) found a parallel line pattern for a similar kinetic study in 100 mM KCl and suggested a sequential mechanism. Because we found the K_m value for phosphite is raised by 100 mM KCl from 0.6 to 1.5 mM (results not illustrated), salt might allow binding of phosphite at both inhibitor and activator sites and thus provide an explanation for the differences in our experiment from that of Withers et al. (1982a). Because phosphite has dual effects, inhibition and activation, on pyridoxal-reconstituted phosphorylase, the kinetic data obtained (Figure 2) might be more complicated than a simple sequential mechanism. To simplify the interpretation of the kinetics, we also used fluorophosphate, another activator anion for the pyridoxal enzyme that showed no inhibition even up to 25 mM (Figure 1), to study the activation process. The resulting double-reciprocal plot showed similar patterns to that of Figure 2, i.e., a set of linear lines converging at the x axis. This observation suggests that a sequential mechanism exists. Fromm's kinetic approach (Fromm, 1964) was used to differentiate between random and ordered mechanisms. Scheme I represents a random mechanism for the addition of glucose-1-P and an activator, phosphite.

The rate equation for this mechanism in the presence of a competitive inhibitor for phosphite (I_b) is

$$\frac{V_{max}}{v} = 1 + \frac{K_{S'}}{[S]} + \frac{K_{A'}}{[A]} + \frac{K_A K_{S'}}{[A][S]} + \frac{K_A K_{S'}[I_b]}{K_{I_b'}[A][S]} + \frac{K_{A'}[I_b]}{K_{I_b}[A]} \quad (1)$$

where E', A, S, K_{I_b} and $K_{I_b'}$ are respectively enzyme complex saturated with glycogen and AMP, phosphite, glucose-1-P, dissocation constant of the inhibitor from I_b–E' complex, and dissociation constant of inhibitor from I_b–E'–glucose-1-P complex. Competitive kinetics with respect to phosphite and noncompetitive kinetics with respect to glucose-1-P are predicted from eq 1. On the basis of the same scheme, the equation in the presence of a competitive inhibitor (I_a) for glucose-1-P can be described as

$$\frac{V_{max}}{v} = \frac{K_{S'}}{[S]} + \frac{K_{A'}}{[A]} + \frac{K_A K_{S'}}{[A][S]} + 1 + \frac{K_A K_{S'}[I_a]}{K_{I_a'}[A][S]} + \frac{K_{S'}[I_a]}{K_{I_a}[S]} \quad (2)$$

where K_{I_a} and $K_{I_a'}$ are the dissocation constants for the interaction of the inhibitor with E' and E'–A complex, respectively. Noncompetitive kinetics with respect to phosphite and competitive kinetics with respect to glucose-1-P are predicted from eq 2. With ordered mechanisms, a competitive inhibitor for the second substrate will yield uncompetitive kinetics with respect to the first substrate. The unique inhibitory pattern permits a distinction to be made between ordered and random bireactant kinetic mechanisms.

Thiosulfate is a competitive inhibitor for phosphite (Figure 3A), but it is a noncompetitive inhibitor with respect to glucose-1-P (Figure 3B). That thiosulfate (up to 20 mM, data not shown) did not affect the enzymatic activity of the native phosphorylase b also indicates that this anion cannot compete with glucose-1-P for the same binding site in the protein. Glucose cyclic 1,2-phosphate is a good competitive inhibitor for glucose-1-P in native phosphorylase (Hu & Gold, 1978). This cyclic compound also acts as a competitive inhibitor for glucose-1-P in pyridoxal-reconstituted phosphorylase b and a noncompetitive inhibitor for phosphite as demonstrated by Withers et al. (1982a). Because no uncompetitive kinetic pattern was observed and the results fit the pattern predicted by eq 1 and 2 derived from Scheme I, the studies support strongly a rapid equilibrium random Bi-Bi mechanism for pyridoxal phosphorylase. The constants, K_A, $K_{A'}$, K_S, and $K_{S'}$, were evaluated from Figure 2 and the same kinetic study with fluorophosphate instead of phosphite, by using eq 1 and 2, and

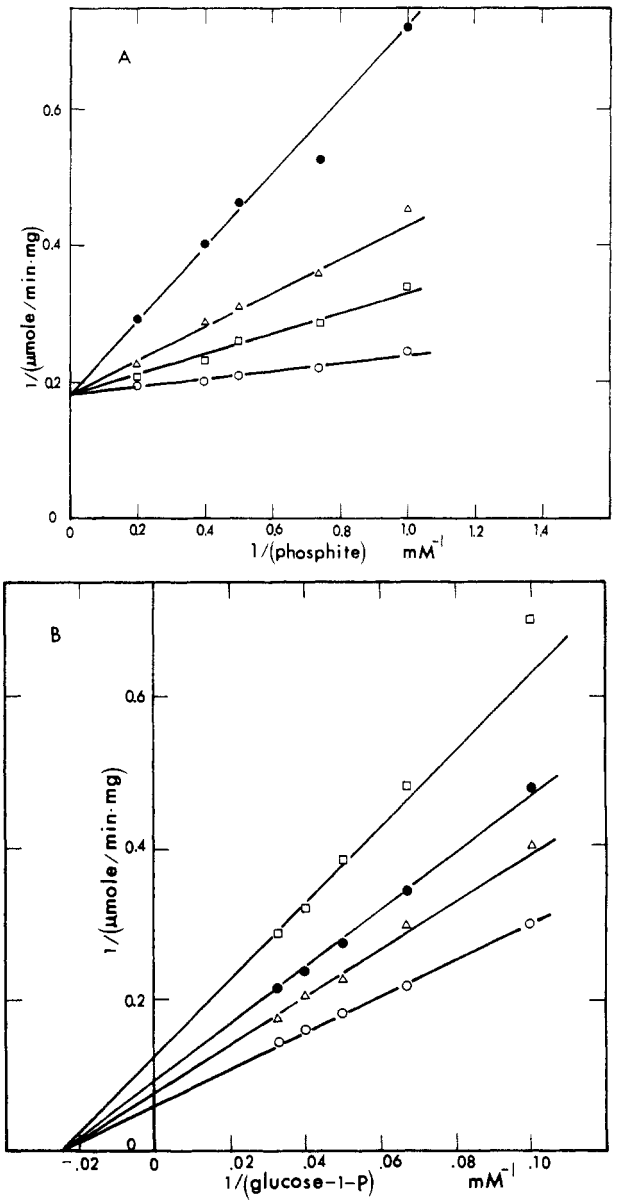


FIGURE 3: Double-reciprocal plots of thiosulfate inhibition of pyridoxal-reconstituted phosphorylase. The reaction mixtures at pH 6.8 and 30 °C consisted of pyridoxal-reconstituted phosphorylase (192 μg/mL), 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, and 1% glycogen. (A) Glucose-1-P at 0.016 M, phosphite at the indicated concentrations, and thiosulfate at 0 (○), 1.0 (□), 2.0 (△), and 4 mM (●). (B) Phosphite at 0.002 M, glucose-1-P at the indicated concentrations, and thiosulfate at 0 (○), 1.0 (△), 2.0 (●), and 4 mM (□).

Table II: Kinetic Parameters of Pyridoxal-Reconstituted Phosphorylase *b* Activated by Phosphite and Fluorophosphate[a]

	phosphite	fluoro-phosphate
V_{max} [μmol/(min·mg)]	15.4	7.7
K_A (mM)	0.8	1.6
$K_{A'}$ (mM)	0.6	1.5
K_S (mM)	18.0	22.3
$K_{S'}$ (mM)	22.0	23.8

[a] Calculations are described under Results and Discussion.

are shown in Table II. That the dissociation constants K_S and K_A are virtually equal to $K_{S'}$ and $K_{A'}$, respectively, shows the binding of activator, either fluorophosphate or phosphite, and glucose-1-P are independent events. If the pyridoxal enzyme–phosphite complex is a good model for coenzyme binding in native phosphorylase, the results also imply that the 5′-phosphoryl group of PLP in native phosphorylase is not needed for glucose-1-P binding, although these two sites are close by in protein. The pyridoxal enzyme–phosphite complex showed weaker affinity for glucose-1-P ($K_{S'}$ = 22 mM) and lower V_{max} (15.4 IU) than those of native phosphorylase [K_m(glucose-1-P) = 7.4 mM and V_{max} = 65 IU; Engers et al., 1969). This observation indicates that the linkage between the phosphoryl group and pyridoxal influence glucose-1-P binding and catalytic capability of phosphorylase. Pfeuffer et al. (1972) showed that PLP molecule is important for the integrity of the native phosphorylase. In the pyridoxal enzyme–phosphite complex, a weak H bond between HPO_3^{2-} and 5-CH_2OH of pyridoxal may partly replace the missing covalent bonding and lead to a recovery of up to 30% of enzymatic activity. In the 5′-deoxypyridoxal-reconstituted phosphorylase,[2] the small activity recovered by phosphite (~0.7% of the native phosphorylase) might be explained by the lack of hydrogen-bond potential between HPO_3^{2-} and the 5-CH_3 on the 5′-deoxypyridoxal molecule. Because the linkage between the phosphoryl group and pyridoxal is important for catalysis and the phosphoryl group of PLP binds strongly to the protein, it is possible that one of the functions of the 5′-phosphoryl group of PLP in phosphorylase is to act as an anchoring point and keep the PLP molecule and/or various amino acid residues in proper position for effective catalysis.

Because the phosphoryl group of PLP was suggested to go through a trigonal-bipyramidal intermediate during phosphorylase catalysis (Withers et al., 1981; Takagi et al., 1982), we used molybdate, which is monomeric (Sasaki et al., 1959) and is believed to be able to mimic the trigonal-bipyramidal state (Van Eteen et al., 1974), in kinetic studies with pyridoxal phosphorylase to further test this mechanism. Molybdate inhibits the pyridoxal enzyme, and kinetic studies show that it is competitive with respect to both phosphite (Figure 4A) and glucose-1-P (Figure 4B). The apparent K_I's of molybdate toward phosphite and glucose-1-P evaluated from secondary plots of Figure 4 are 0.1 and 2.5 mM, respectively. Because molybdate binds more tightly to the PLP phosphite site on the enzyme than phosphite (K_m = 0.6 mM), this result suggests that this oxyanion may resemble the transition state of the enzyme-bound phosphite during the catalysis. This transition state that molybdate imitates may be a trigonal-bipyramidal species as suggested by Van Eteen et al. (1974). Vanadate and tungstate also can mimic the trigonal-bipyramidal state (Van Eteen et al., 1974) and are inhibitors of pyridoxal phosphorylase. The exact interpretation of the results is more

[2] R. F. Parrish and D. J. Graves, unpublished results.

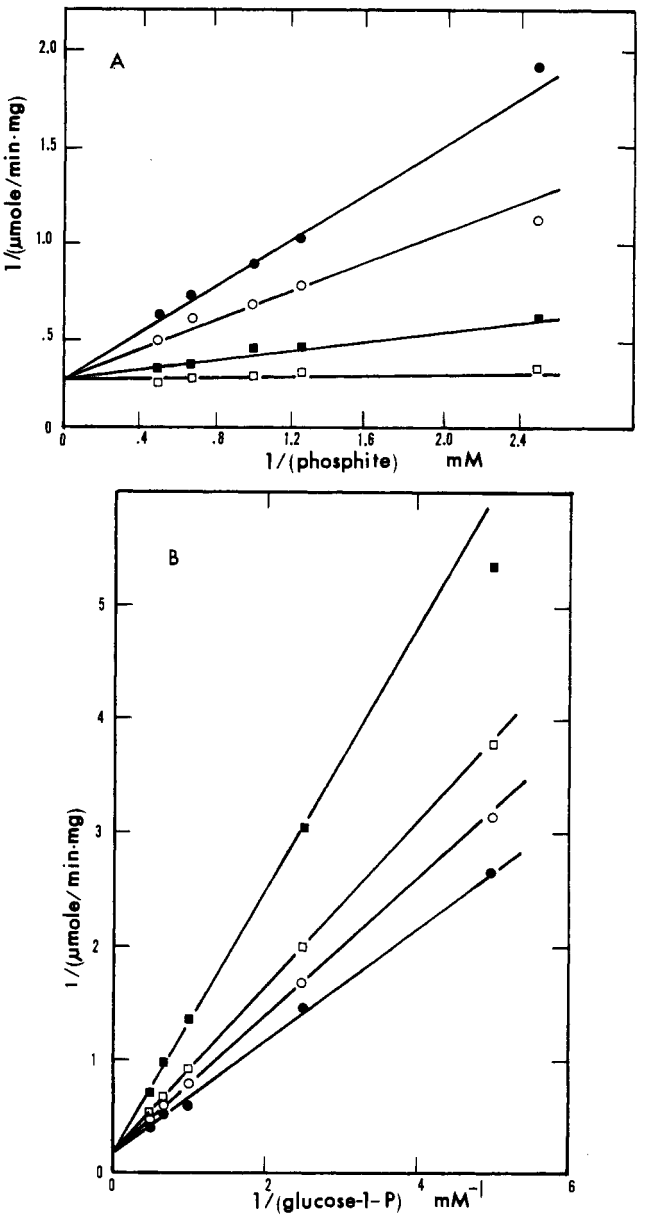


FIGURE 4: Double-reciprocal plots of molybdate inhibition of pyridoxal-reconstituted phosphorylase *b*. Reaction mixtures at pH 6.8 and 30 °C consisted of 0.04 M β-glycerophosphate, 0.002 M EDTA, 0.001 M AMP, 1% glycogen, and 225 μg/mL pyridoxal-reconstituted phosphorylase *b*. (A) [U-^{14}C]Glucose-1-P at 0.016 M, phosphite at the indicated concentrations, and molybdate at 0 (□), 1.25 (■), 3.0 (○), and 5 mM (●). (B) Phosphite at 0.002 M, [U-^{14}C]glucose-1-P at the indicated concentrations, and molybdate at 0 (●), 1.0 (○), 2.0 (□), and 5.0 mM (■).

complex because these oxyanions can exist in different oligomeric states. An account of their effects on phosphorylase is found in the accompanying paper (Soman et al., 1983). The possibility that the enzyme-bound phosphate is transformed into a trigonal-bipyramidal intermediate during the catalysis is also supported by the observation of the varied effects of phosphate analogues with pyridoxal phosphorylase. When one oxygen atom on the phosphate is substituted by an electron-donating group such as sulfur, methyl, or ethyl, these anions show lower V_{max}'s than does phosphate (see Table I). The low electrophilicity of the center phosphorus atom in these analogues makes them harder to form the trigonal-bipyramidal intermediate needed for the catalysis and might be responsible for their low V_{max} values. On the basis of the differences of effects (K_m and V_{max}) between phosphate analogues and sulfate

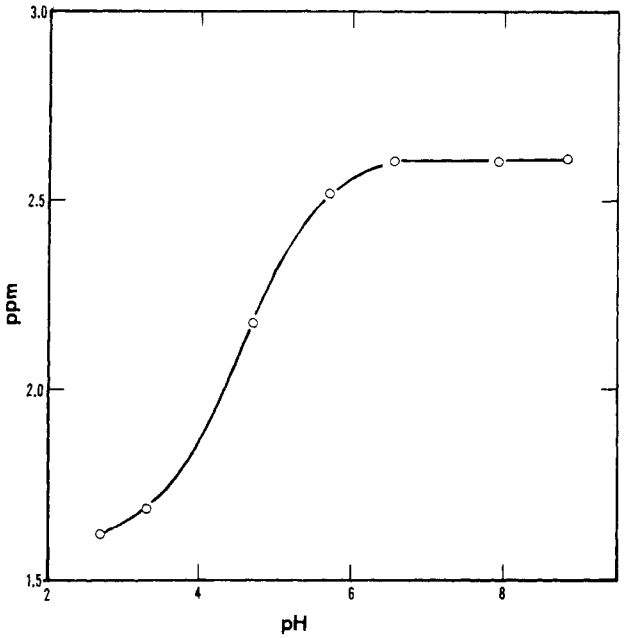


FIGURE 5: ^{19}F NMR chemical shift, average of the doublet in each ^{19}F NMR spectrum, of fluorophosphate at different pH values. Conditions were as described under Materials and Methods.

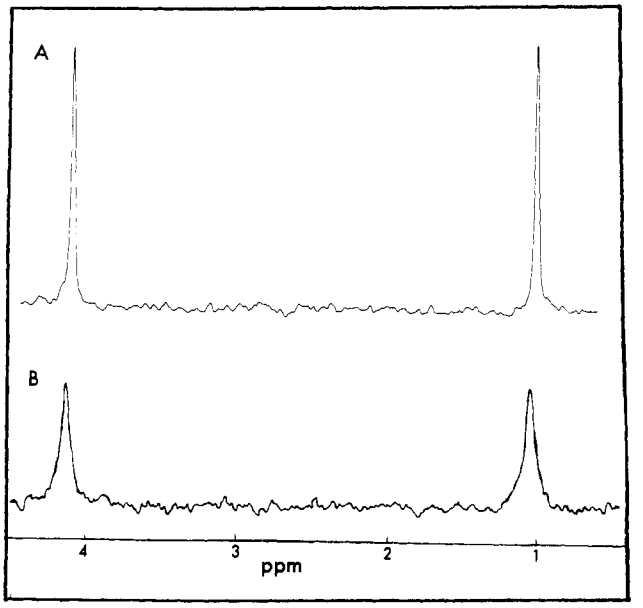


FIGURE 6: (A) Fluorine magnetic resonance spectrum of 0.00023 M fluorophosphate in a solution at pH 6.8 consisting of 0.04 M β-glycerophosphate, 0.03 M 2-mercaptoethanol, 0.001 M AMP, and 0.002 M EDTA, 18 064 acquisitions. (B) Fluorine nuclear magnetic resonance spectrum of a similar solution as in (A) with added pyridoxal-reconstituted phosphorylase *b* (21.7 mg/mL), 18 064 acquisitions. Conditions were as described under Materials and Methods.

analogues, the nature of the center atom seems to be important for their effects on pyridoxal enzyme. Thiophosphate is an activator, but thiosulfate can bind to the protein and inhibit the restoration of enzymatic activity by phosphite. Phosphate can activate, but sulfate (at 1–5 mM), because it does not activate or inhibit, probably does not even bind to the pyridoxal enzyme. These differences can be explained by the fact that phosphorus can more easily expand its valence shell to form a trigonal-bipyramidal structure than sulfur.

Although the results with molybdate and varied phosphate analogues are consistent with a mechanism that a trigonal-bipyramidal intermediate is involved in the catalysis, results in this study are different from those reported previously in other systems. When molybdate was used as a transition-state analogue in phosphatases, it binds to the enzyme 10^3–10^4 times more tightly than phosphate, while the binding of molybdate to pyridoxal enzyme is only 13 times tighter than phosphate. The variation in V_{max}'s among phosphate and its analogues, thiophosphate and methyl- or ethylphosphonate, in this study (see Table I) is much smaller than those found in hydrolytic reactions among phosphoester and its analogues (Benkovic & Schray, 1973), which were believed to proceed through a trigonal-bipyramidal intermediate. These differences could be explained by either that the structural transformation of PLP phosphate into a trigonal-bipyramidal species is not the rate-limiting step during the catalysis or that the hypothesis that PLP phosphate forms a trigonal-bipyrimidal intermediate is not a perfect description of the real mechanism of phosphorylase. Therefore, other mechanisms with the involvement of PLP in the catalysis of phosphorylase cannot be excluded.

Because fluorophosphate is a good activator of pyridoxal phosphorylase and has a low pK_a value (4.8), but the pyridoxal enzyme activated by fluorophosphate does not have a different pH profile than that of phosphate, Withers et al. (1982b) suggested that it is unlikely that the phosphoryl group of PLP in phosphorylase is involved in a proton shuttle. However, if a change in the pK_a value of fluorophosphate occurred upon binding, a similar pH profile might be obtained for the enzyme activated by the two anions—even if the phosphoryl group was involved in a proton-shuttle mechanism. ^{19}F NMR spectroscopy was used to examine different ionization states of fluorophosphate. Because the fluorine nucleus is coupled by the adjacent phosphorus ($I = 1/2$), the ^{19}F NMR spectrum of fluorophosphate shows a doublet. ^{19}F NMR spectra of fluorophosphate have been followed at varied pH's, and the averages of the chemical shifts of the doublets are shown in Figure 5. A transition of the ^{19}F resonance in Figure 5 is found between pH 2 and 6, and the midpoint of this transition is measured to be 4.5, which corresponds to the pK_a of fluorophosphate determined by other methods (Van Wazer, 1958). ^{19}F NMR spectra of 0.23 mM fluorophosphate in the absence and presence of an equal concentration of pyridoxal phosphorylase *b* have been obtained and are shown in Figure 6. The broadening of ^{19}F NMR signals of free fluorophosphate upon addition of the enzyme indicates that part of fluorophosphate is bound to the protein. The two sets of resonances in Figure 6 have identical integration of their areas relative to the external referencing signal (TFA). The fluorophosphate signals shifted slightly downfield (~0.04 ppm) in the presence of enzyme. This difference might be explained by the differences in susceptibility of the external standard or some mechanism other than a protonation process, which would shift the signals upfield. This observation shows that the binding to the pyridoxal enzyme does not change the ionization state of fluorophosphate. Phosphate (58 mM), another activator, which was added to the fluorophosphate and enzyme mixture to release the bound fluorophosphate from the enzyme, caused the ^{19}F signals to become the same width as the signal obtained from free fluorophosphate, while the area under the ^{19}F signals remained unchanged. Mixing fluorophosphate (1 mM) with enzyme (0.2 mM), AMP (1 mM), glucose-1-P (15 mM), and maltopentaose (1%) causes the half-height width of the ^{19}F signals to decrease from 19.9 Hz (measured at 8 min after mixing) to a steady value of 13 Hz (after 20 min), while the chemical shift and the areas of ^{19}F resonance measured at different times remained unchanged. The gradual narrowing of ^{19}F signals as the reaction proceeded indicates that the increasing amount of phosphate produced can release fluo-

rophosphate from the protein by competing for the same binding site on protein. Because no detectable change of the fluorophosphate-signal positions occurred during catalysis, the results indicate that no appreciable change in the ionization state of bound fluorophosphate happens during the reaction. However, it is possible that a proton shuttle might not be detected by NMR spectroscopy if the steady-state concentration of a protonated enzyme form, which may exist transiently during the catalysis, is low.

In summary, the data in this study are consistent with a mechanism in which the 5′-phosphoryl group of PLP forms a trigonal-bipyramidal intermediate during the catalysis. Phosphate analogues bound to the pyridoxal phosphorylase, and possibly the 5′-phosphoryl group of PLP in native enzyme, are unlikely to undergo a protonation–deprotonation process. Kinetic study indicates that glucose-1-P does not need the phosphoryl group of PLP for its binding to phosphorylase. The dianionic state of the PLP phosphate is crucial for this coenzyme to perform its functions, one of which could be holding a proper active site region for effective catalysis. Other functions of PLP in phosphorylase are still possible and need to be further explored.

Registry No. Glucose-1-P, 59-56-3; PLP, 54-47-7; phosphite, 14901-63-4; fluorophosphate, 15181-43-8; phosphate, 14265-44-2; thiophosphate, 15181-41-6; (aminomethyl)phosphonate, 14047-31-5; ethylphosphonate, 16486-09-2; methylphosphonate, 16002-10-1; selenite, 14124-67-5; sulfite, 14265-45-3; thiosulfate, 14383-50-7; arsenate, 15584-04-0; molybdate, 11116-47-5; phosphorylase *b*, 9012-69-5.

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