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^{31}P nuclear magnetic resonance studies of glycogen phosphorylase from rabbit skeletal muscle: Ionization states of pyridoxal 5′-phosphate*

(thio analogues of adenosine nucleotides/allosteric activation/interconversion/active conformation)

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ABSTRACT **^{31}P nuclear magnetic resonance (NMR) at 72.8 MHz has been used to study glycogen phosphorylase from rabbit muscle (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) at concentrations as low as 25 mg/ml, using a WH-180 wide-bore superconducting spectrometer. The use of a thio analogue for 5′-AMP and arsenate for inorganic phosphate allowed the observation of three distinct forms of enzyme-bound pyridoxal 5′-phosphate at −0.2 ppm (Form I), −2 to −3 ppm (Form II), and −3.5 ppm (Form III) relative to triethylphosphate. Conversion of I to III occurs by activation of phosphorylase either by formation of a ternary complex of phosphorylase *b* with effector and arsenate or, more efficiently, by direct phosphorylation to give the *a* form of the enzyme. The ionization state and exposure to solvent of each of the three forms is inferred from the ^{31}P NMR data.**

Pyridoxal 5′-phosphate is an essential constituent of all known α-glucan phosphorylases (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1). Reconstitution experiments with pyridoxal-*P* analogues (1–3) indicate the importance of the phosphate moiety in pyridoxal-*P* function but do not provide information on its precise role (4, 5). ^{31}P nuclear magnetic resonance (NMR) spectroscopy gives specific information on the ionization state and the pK_a values of pyridoxal-*P* bound to the enzyme. In addition, one can observe all other phosphorus-containing ligands involved in phosphorylase catalysis, as well as the phosphoserine group that regulates the activity of phosphorylase by interconversion of the *b* and *a* forms (6). However, the ^{31}P NMR signals for P_i, glucose 1-phosphate, AMP, phosphoserine, and pyridoxal-*P* all lie in a very narrow range of *ca* 4 ppm. Therefore, we have substituted adenosine 5′-*O*-thiomonophosphate (AMP-S) for AMP and arsenate for phosphate. Adenosine 5′-*O*-(3-thio)triphosphate (ATP-γ-S) was used to prepare phosphorylase *a* containing thiophosphoserine residues. Thus, it was possible to separate the signals of the various ligands and to clearly observe the pyridoxal-*P* resonance, which monitors the protein conformational change induced by the effector AMP or by the phosphoserine group.

MATERIALS AND METHODS

Phosphorylase *b* was prepared from frozen rabbit skeletal muscle (7) and recrystallized at least three times before use. It was converted to the thio analogue of phosphorylase *a* by phosphorylase kinase (EC 2.7.1.38), Mg^{2+}, and ATP-γ-S according to Gratecos and Fischer (8). Phosphorylase *a* containing the thiophosphoseryl residue has the same activity as the normal phosphorylase *a* (8). Enzymatic activities were measured in the direction of glycogen synthesis (9) as described previously (10). Protein concentrations were determined using $A_{280}^{1\%}\,cm^{-1} = 13.2$ (4). Molar concentrations are based on a molecular weight of 100,000 for each monomer (11). AMP was removed by passage over a Sephadex G-25 column (2.5 × 80 cm) or by treatment with activated charcoal. Buffer A is 100 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), 2 mM EDTA, 50 mM 2-mercaptoethanol. The pH was adjusted as desired with NaOH. Buffer B is 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM EDTA, pH 7.76 (12). Buffer chemicals were from Sigma; sugar phosphates and nucleotides, including the thio analogues, were from Boehringer & Sons; arsenate, oyster glycogen, and charcoal were from E. Merck. Glycogen was freed from AMP as described previously (13). The pH of each sample was measured before and after the NMR measurements with a Knick 641 digital pH meter and a Radiometer electrode GK 2321C, calibrated with two standard buffers.

^{31}P Fourier-transformed NMR spectra were recorded at 72.8 MHz with a Bruker WH-180 wide-bore superconducting spectrometer. Sample volumes were 10–15 ml in 20 mm diameter sample tubes. A concentric 5 mm NMR tube containing D_2O was used for field/frequency lock. It was replaced by a tube containing *ca* 0.5 M triethylphosphate in D_2O for chemical shift referencing. All spectra were recorded with proton noise decoupling (1 W, *ca* 500 Hz bandwidth). The 90° pulse width in aqueous solutions is *ca* 50–60 μsec. For enzyme samples the exponential linebroadening used before Fourier transformation was usually 10 Hz. All linewidth data have been corrected for this linebroadening effect. Continuous air flow through the spectrometer probe head maintained the temperature between 28 and 30°.

RESULTS

Interaction of Phosphorylase *b* with the Allosteric Effector AMP. Phosphorylase contains one covalently bound pyridoxal-*P* per monomer. Because the enzyme preparations were recrystallized with AMP, the nucleotide was carefully removed prior to NMR studies (*Materials and Methods*). Fig. 1*A* gives the ^{31}P NMR spectrum of the cofactor bound to phosphorylase *b*. In Fig. 1*B* a stoichiometric amount of one AMP per monomer has been added, and in Fig. 1*C* the sample contains two molecules of AMP per monomer and 100 mM arsenate. Addition of AMP alone at stoichiometric levels does not affect the chemical shift of pyridoxal-*P* (Fig. 1*B*). AMP shows one exchange-averaged signal for the free and bound forms. The addition of arsenate sufficiently tightens the binding of AMP (14, 15) so that slow-to-intermediate exchange conditions apply, and the

Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; Pxy-*P* and pyridoxal-*P*, pyridoxal 5′-phosphate; AMP-S, adenosine 5′-*O*-thiomonophosphate; ATP-γ-S, adenosine 5′-*O*-(3-thio)triphosphate.

* This is part of a series; the preceding paper is ref. 15.

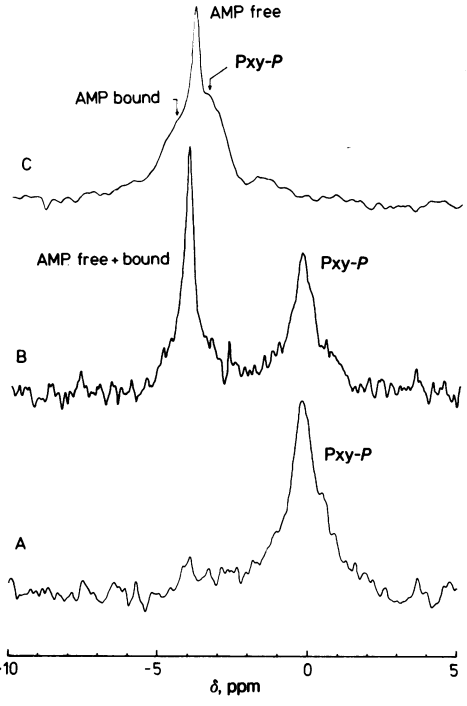

FIG. 1. ^{31}P NMR spectra of phosphorylase *b* with and without AMP and arsenate. (*A*) Phosphorylase *b* free of AMP at 50 mg/ml (0.5 mM monomers) in buffer B, pH 7.76. The chemical shift of the pyridoxal phosphate (Pxy-*P*) is −0.23 ppm. The spectrum represents 109,000 scans with acquisition time 0.34 sec and pulse width 30 μsec. (*B*) Phosphorylase *b* 0.4 mM monomers and 0.4 mM AMP in buffer A, pH 8.2. The chemical shifts of pyridoxal-*P* and AMP are −0.29 and −4.14 ppm, respectively. The spectrum represents 60,000 scans with acquisition time 0.568 sec and pulse width 25 μsec. (*C*) Phosphorylase *b* 0.4 mM monomers, 0.8 mM AMP, and 100 mM arsenate in buffer A, pH 7.64. The chemical shifts of bound AMP, free AMP, and pyridoxal-*P* are estimated to be −4.4, −4.01, and −3.6 ppm. The spectrum represents 40,500 scans with acquisition time 0.34 sec and pulse width 30 μsec.

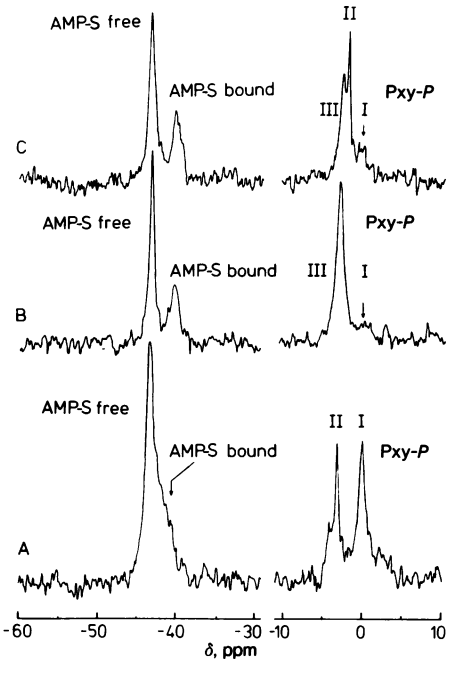

FIG. 2. ^{31}P NMR spectra of phosphorylase *b* in the presence of AMP-S and arsenate. (*A*) Phosphorylase *b*, 0.52 mM monomers with 1.45 mM AMP-S in buffer A, pH 7.0. The chemical shift of free AMP-S in exchange with bound AMP-S is −43.4 ppm. Pyridoxal-*P* is split into two resonances, I and II, with shifts of −0.16 and −3.33 ppm, respectively. The spectrum represents 11,200 scans with a repetition time of 1.3 sec and pulse width 30 μsec. (*B*) Conditions as in *A* but with 36 mM arsenate. The shifts for free and bound AMP-S are −43.7 and −40.5 ppm, respectively. Pyridoxal-*P* gives resonance III at −3.48 ppm and a small amount of I at −0.2 ppm. The spectrum represents 23,700 scans with repetition time 1.3 sec and pulse width 30 μsec. (*C*) Conditions as in *B* but with pH lowered to 6.4 with acetic acid. The chemical shifts of free and bound AMP-S are −43.5 and −40.5 ppm, respectively. The resonances I, II, and III for pyridoxal-*P* have shifts of *ca* −0.6, −2.60, and 3.28 ppm, respectively. The spectrum represents 9300 scans with repetition time 1.3 sec and pulse width 30 μsec.

resonances of free and bound AMP are partially resolved (Fig. 1*C*). The arsenate-induced tightening of AMP binding results in a shift of the pyridoxal-*P* resonance from its position in the absence of arsenate at *ca* −0.2 ppm to a new position of *ca* −3.6 ppm. Arsenate alone has only a minor effect on the pyridoxal-*P* resonance (spectrum not shown, compare Fig. 3*A*). In Fig. 1*C* the difference between the free and bound AMP chemical shifts is $\Delta = \delta_{free} - \delta_{bound} = 0.4$ ppm. The slow exchange condition specifies an upper limit ($k_{-1} < 2\pi\Delta$) of 180 sec^{-1} for the dissociation rate constant k_{-1} of AMP and phosphorylase *b* when arsenate is present. In the absence of arsenate (fast exchange case, Fig. 1*B*) the exchange-averaged chemical shift of the AMP resonance is

$$\delta_{obs} = \delta_{free} - \Delta\{[\text{E-AMP}]/[\text{AMP}]_0\} \qquad [1]$$

in which [E-AMP] and $[AMP]_0$ are the concentrations of enzyme-bound and total AMP. Thus, in Fig. 1*B* [E-AMP] = 0.13 mM and the dissociation constant K_d for AMP and phosphorylase *b* in the absence of arsenate is *ca* 0.5 mM. This agrees well with the K_a values for AMP and phosphorylase *b* in the absence of substrates (4, 14, 16).

Interaction of Phosphorylase *b* with the Allosteric Effector AMP-S. The difficulty in resolving AMP and pyridoxal-*P* resonances was overcome by using AMP-S, an even better activator (17).

In Fig. 2*A*, AMP-S (2.8 mol/mol of monomer) has been added to phosphorylase *b* free of AMP, pH 7.0. The free AMP-S is in intermediate exchange (near coalescence) with AMP-S bound to phosphorylase *b* and $k_{-1} \approx 2\pi\Delta = 1.5 \times 10^3$ sec^{-1}. The pyridoxal-*P* resonance is split into two forms. Form I is that seen with phosphorylase *b* alone with linewidth $\Delta\nu = 70$ Hz. Form II is distinguishable from form I by chemical shift and linewidth $\Delta\nu = 30$ Hz. In Fig. 2*B* arsenate has been added, pH 7.1. Analogous to the effect shown with AMP (Fig. 1*C*) the binding of AMP-S is tightened and pyridoxal-*P* is now almost completely in form III, $\Delta\nu = 70$ Hz. In Fig. 2*C* at lower pH all three pyridoxal-*P* forms are detectable.

In Fig. 3 phosphorylase *b* was titrated with AMP-S in the presence of 50 mM arsenate, pH 7.8–8.1. Slow-exchange conditions apply for both AMP-S and pyridoxal-*P*. The conversion of form I to form III of pyridoxal-*P* parallels the increase in bound AMP-S. Because exchange is likely to be faster than the T_1 relaxation times (estimated to be 1–5 sec), the relative amounts of free and bound AMP-S and of form I and III of pyridoxal-*P* may be determined in any one spectrum by measuring the peak areas, independent of pulsing conditions (18). Thus, one can estimate from spectra *B*, *C*, and *D* in Fig. 3 that the values of $K_d = k_{-1}/k_1$ for the AMP-S–phosphorylase *b* complex in the presence of arsenate are *ca* 0.1 mM, depending on AMP-S concentrations, in agreement with published values for K_a of AMP and phosphorylase *b* in the presence of phos-

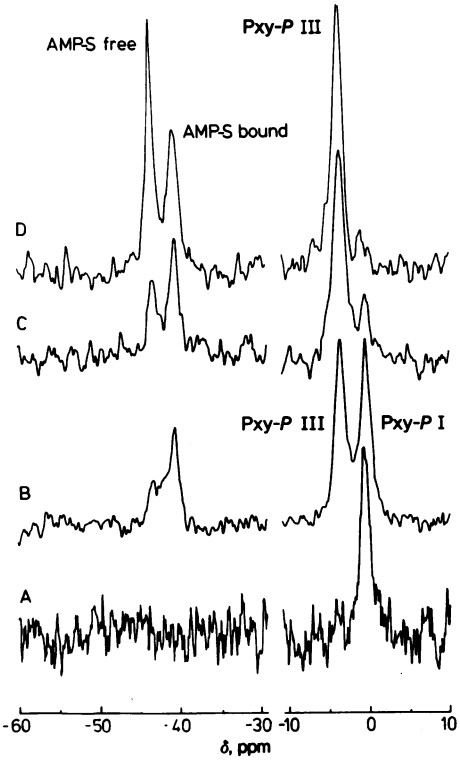

FIG. 3. ^{31}P NMR spectra of phosphorylase *b* titrated with AMP-S in the presence of arsenate. (*A*) Phosphorylase *b*, 0.62 mM monomers, free of endogenous AMP, with 50 mM arsenate in buffer A, pH 7.75. The chemical shift of pyridoxal-*P* is −0.37 ppm. The spectrum represents only 4300 scans with repetition time 1.3 sec and pulse width 30 μsec. (*B*) Conditions as in *A* but with 0.38 mM AMP-S, pH 8.0. The chemical shifts are: free AMP-S, −43.3 ppm; bound AMP-S, −40.6 ppm; form I of pyridoxal-*P*, −0.47 ppm; form III of pyridoxal-*P*, −3.67 ppm. The spectrum represents 27,000 scans with the same conditions as in *A*. (*C*) The AMP-S concentration was 0.75 mM (1.21 molecules/monomer), pH 8.0, 8500 scans. (*D*) The AMP-S concentration was 1.08 mM (1.74 molecules/monomer), pH 8.1, 9900 scans. The vertical scales in these spectra are not directly comparable.

phate or arsenate (14). Assuming a simple bimolecular association reaction at a unique binding site, the linewidth $\Delta\nu$ of free AMP-S is given by

$$\pi\Delta\nu = \pi\Delta\nu_0 + k_{-1}\{[\text{E-AMP-S}]/[\text{AMP-S}]\} \quad [2]$$

in which $\Delta\nu_0$ is the linewidth of the free AMP-S in the absence of exchange and [E-AMP-S] and [AMP-S] are the bound and free AMP-S concentrations. From the linewidths for free AMP-S in Fig. 3 one estimates a value of k_{-1} of 200 ± 50 sec^{-1}. The linewidths of forms I and III of pyridoxal-*P* place a strict upper limit of 250 sec^{-1} for the interconversion of these two species.

Interaction of ATP with Phosphorylase *b*. ATP is known to be a competitive inhibitor with respect to AMP activation of phosphorylase *b* (19). The ^{31}P NMR spectrum was recorded for a sample with 38 mg/ml of phosphorylase *b* (free of AMP), 50 mM arsenate, and 5 mM ATP in buffer A, pH 7.3. A dissociation constant of ATP and phosphorylase *b* in the absence of substrate of 2.0 and 2.8 mM has been determined (14, 20). Since the K_i depends on the concentration of the anionic substrate P_i, the dissociation constant of ATP in the presence of 50 mM arsenate should be <1 mM (14). Thus, at least 80% of the enzyme nucleotide sites are occupied under the above conditions; however, no effect on the pyridoxal-*P* resonance was observed and form III was not detected.

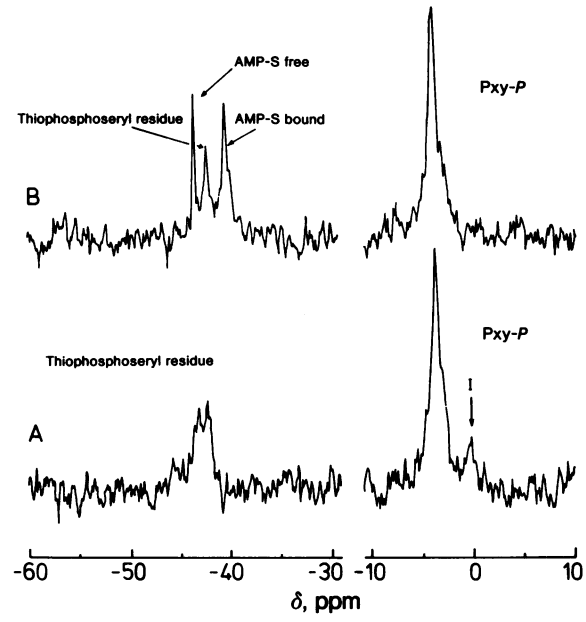

FIG. 4. ^{31}P NMR spectra of thiophosphoseryl phosphorylase *a*. (*A*) Thiophosphoseryl phosphorylase *a* at 25 mg/ml (0.25 mM monomers) in buffer A containing 0.3 M NaCl (cf. ref. 26) at pH 7.67. The chemical shifts are: form I, −0.22 ppm; form III, −3.76 ppm; thiophosphoserine residue, −43.0 and −42.3 ppm. The spectrum represents 28,300 scans with parameters as in Fig. 3. (*B*) AMP-S (1.39 molecules/monomer) was added to the sample. The shifts are: free AMP-S, −43.6 ppm; bound AMP-S, −40.5 ppm; thiophosphoserine residue, −42.3 ppm; form III of pyridoxal-*P*, −3.76 ppm. The spectrum represents 21,400 scans.

Phosphorylase *a*. Phosphorylase *a* containing thiophosphoseryl residues was prepared (*Materials and Methods*). Judging by activity, this reaction was at least 80% complete. The ^{31}P NMR spectrum of the thiophosphoseryl-phosphorylase *a* at pH 7.7 is shown in Fig. 4*A*. The major pyridoxal-*P* resonance is virtually identical with that found for the phosphorylase *b*–AMP-S complex (compare Figs. 3*C* and 4*A*). The small amount (17%) of form I of pyridoxal-*P* is probably due to contamination with nonconverted phosphorylase *b*. The thiophosphoserine apparently has two peaks at pH 7.7 but only one of these (*ca* −42.2 ppm) is observed in the presence of AMP-S (Fig. 4*B*) or at pH 6.3 (spectrum not shown).

AMP-S was added to the sample as shown in Fig. 4*B*. The form III resonance remained unchanged, the small amount of form I disappeared as expected, and the thiophosphoserine now gave a sharper resonance at −42.3 ppm. From the peak areas the amount of AMP-S bound to phosphorylase *a* was calculated to be 1.0 ± 0.1 mol/mol of monomer. Thus, the AMP binding sites were free to bind the stoichiometrically expected amount of AMP-S. The linewidths of free and bound AMP-S were 10 and 35 Hz, respectively. Using Eq. 2 and assuming $\Delta\nu_0$ = 1 Hz for free AMP-S, k_{-1} is estimated to be 11 sec^{-1}.

The pH Dependence of the Chemical Shifts of Pyridoxal-*P*, AMP, and AMP-S. Fig. 5 summarizes the chemical shifts of pyridoxal-*P* bound to phosphorylase *b* as a function of pH and ligands. Because pyridoxal-*P* is bound as a Schiff base to a lysyl side chain of phosphorylase (15), the pH dependence of the model Schiff base formed from pyridoxal-*P* and *N*-α-acetyl-L-lysine methyl ester is shown for comparison (broken line in Fig. 5). The pK_a values for the model Schiff base and for free pyridoxal-*P* were both 6.1 under comparable conditions, although their chemical shifts at high pH are significantly different (pyridoxal-*P*, −4.15 ppm; Schiff base, −3.81 ppm).

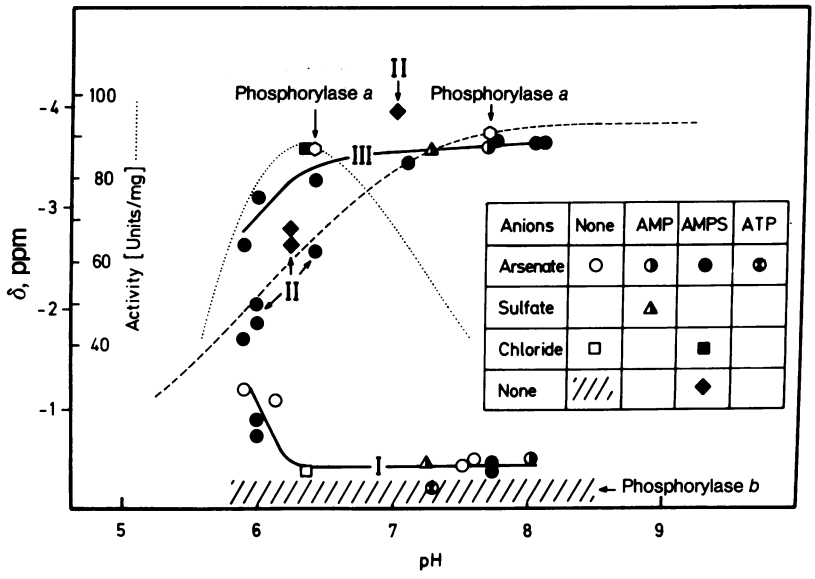

FIG. 5. pH dependence of ^{31}P chemical shifts of pyridoxal-*P* bound to phosphorylase. The symbols refer to the additions of anions and/or effectors in buffer A. Three forms of pyridoxal-*P* have been characterized (compare Fig. 2) and are denoted by the numerals I, II, and III. In addition, two data points for phosphorylase *a* have been given symbol ○. The hatched region represents the range of chemical shifts for phosphorylase *b* in the absence of ligands. The broken line shows the pH dependence of the ^{31}P resonance of a Schiff base formed between pyridoxal-*P* and *N*-α-acetyl-L-lysine methyl ester. The samples contained 0.3–0.4 mM phosphorylase *b*. Concentration of arsenate was 36–100 mM, of sulfate 100 mM, of chloride 0.3 M. Superimposed on this plot is the activity profile (dotted curve) for phosphorylase *b* in buffer A instead of 100 mM maleate. Activity is given in units/mg (cf. ref. 10).

In the absence of the anionic substrate analogue the phosphorus resonance of form I of pyridoxal-*P* bound to phosphorylase *b* was pH-independent between pH 5.8 and 8.5 (hatched region in Fig. 5). Addition of high concentrations of arsenate, sulfate, or chloride caused a small but consistent downfield shift of the phosphorus resonance of form I. Addition of AMP or AMP-S resulted in the conversion of form I to form III with a chemical shift corresponding to the doubly ionized Schiff base. Both form I and form III become sensitive to pH only below pH 6.4, and this change correlates with the point of maximal enzyme activity (Fig. 5). Below pH 7 the form II could be observed to have a pH dependence similar to that of the model Schiff base.

In the pH range 7.3–8 the chemical shift of AMP is −4.0 ppm (dianion) and moves upfield as the pH is lowered, as observed for other phosphate esters (21, 22). The resonance of AMP bound to phosphorylase is shifted downfield *ca* 0.4 ppm from the position of the free dianion. Because of the strong influence of the thio group, AMP-S behaves in the opposite sense, with binding producing an upfield shift of *ca* 3 ppm.

DISCUSSION

With the exception of glycogen phosphorylases, other enzymes that utilize pyridoxal-*P* in catalysis are inactivated by $NaBH_4$ reduction of the Schiff base linkage (23). Chemical modification studies have directed attention to the phosphate group of pyridoxal-*P* (4, 5, 15, 24). This phosphate is not transferred during catalysis (1), but its role as a proton donor/acceptor is suggested by the fact that maximal enzyme activity occurs at a pH very near the pK_a of pyridoxal-*P* for native phosphorylase and near the pK_a of pyridoxal 5′-methylenephosphonate for the corresponding phosphorylase derivative (24).

Three distinct forms of enzyme-bound pyridoxal-*P* have been characterized. These species are interconverted by the allosteric transition induced by AMP, AMP-S, or phosphorylation of the enzyme. ATP is a competitive inhibitor with respect to AMP, which does not activate phosphorylase *b*. Accordingly, ATP does not affect the pyridoxal-*P* resonance. High concentrations of the anions arsenate, sulfate, and chloride tighten the binding of the allosteric effectors AMP and AMP-S, thus facilitating the interconversion of the pyridoxal-*P* forms.

The pH-insensitive chemical shifts of the forms III and I are consistent with the respective ionization states $R\text{-}O\text{-}P(O)O_2^{2-}$ and $R\text{-}O\text{-}P(O)O_2H^-$ or possibly $R\text{-}O\text{-}P(O)O_2H_2$. Form I is observed only in inactive native phosphorylase *b* in the absence of effectors. Form III is observed only in the active tight phosphorylase *b*–effector complex in the presence of arsenate or in phosphorylase *a*. Hence a plausible explanation is that the allosteric transition from inactive to active enzyme results in the deprotonation of the phosphate group within a protective enzyme binding site. This interpretation, however, relies on the assumption that the interaction of the phosphate with the enzyme does not by itself produce a significant perturbation in the chemical shift. The binding of AMP to phosphorylase at pH 7.6 (presumably as dianion) results in a downfield shift of *ca* 0.4 ppm.

The tight binding of P_i to alkaline phosphatase of *Escherichia coli* likewise results in a downfield shift of *ca* 1 ppm (to −3.5 ppm) at pH 8 (18). This bound P_i was insensitive to pH just as is form III of pyridoxal-*P* in phosphorylase *b*. Thus, we tentatively conclude that form III represents a "normally" bound phosphate dianion, and that form I of pyridoxal-*P* in the inactive or "switched-off" enzyme is a unique, possibly protonated, species. This conclusion differs from that of Busby *et al.* (12).

At pH below 7 a third pyridoxal-*P* species was identified (form II), which had a narrower linewidth (30 Hz). Its pH dependence was like that of the Schiff base formed between *N*-α-acetyl-L-lysine methyl ester and pyridoxal-*P*. In this form the phosphate group is free to rotate (line-narrowing) and to interact with the solvent. At pH 7.2, form II cannot be detected when form III is also present due to the equivalence of chemical shifts (Fig. 2*B*), but in the absence of anions (Fig. 2*A*) form II instead of III was observed in the presence of AMP-S. Addition

of free pyridoxal-*P* showed that form II is not the free cofactor. Denaturation of protein was also unlikely, judged on activity. Interestingly, form II was not detected in phosphorylase *a* at pH 6.3. Thus, the appearance of II may result from a loose binary complex of AMP-S and enzyme (Fig. 2*A*). The formation of the more stable ternary complex with arsenate produces form III in phosphorylase *b*. Form III prevails in phosphorylase *a* without effector and anions, correlating with the fact that phosphorylase *a* is active in the absence of the effector. For comparison, cytoplasmic pig heart aspartate aminotransferase (EC 2.6.1.1) appears to bind pyridoxal-*P* exclusively as the dianion (25).

The natural linewidth (without exchange) for AMP-S bound to phosphorylase *a* is *ca* 30 Hz. This suggests an exchange linewidth of 50–70 Hz for AMP-S bound to phosphorylase *b*, giving a dissociation rate k_{-1} of 160–220 sec^{-1}, in good agreement with the value of 200 ± 50 sec^{-1} deduced from the concentration dependence of the free AMP-S linewidth. Thus, estimated values of the dissociation rate k_{-1} for AMP-S from phosphorylase *a* and from phosphorylase *b* with and without arsenate are *ca* 10, 200, and 1500 sec^{-1}, respectively. Estimates for the dissociation constant K_d are: for AMP and phosphorylase *b* without arsenate, 0.5 mM; for AMP-S and phosphorylase *b* with arsenate, 0.1 mM. Since forms I and III of pyridoxal-*P* have similar linewidths under a variety of conditions, the exchange linewidth is probably small, implying first-order rate constants of less than 100 sec^{-1} for the interconversion of these forms.

An involvement of pyridoxal-*P* in the allosteric transition of phosphorylase has been demonstrated, but whether the protons leaving the phosphate group actually participate in general acid base catalysis or are transferred to an amino acid residue at the active site is still a matter of speculation.

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