provided excellent technical assistance.

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Covalently Activated Glycogen Phosphorylase: A Phosphorus-31 Nuclear Magnetic Resonance and Ultracentrifugation Analysis†

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ABSTRACT: Glycogen phosphorylase *b* reconstituted with pyridoxal pyrophosphate in place of the natural coenzyme, pyridoxal phosphate, is shown to exist in a more activated (R) conformation than does native phosphorylase. Addition of nucleotide activator to the reconstituted enzyme traps it totally in this activated conformation. These conclusions were arrived at on the basis of tertiary structural information obtained from ^{31}P nuclear magnetic resonance studies, which allowed measurement of the nucleotide binding constant, and on the basis of quaternary structural information obtained via ultracentrifugal analysis of the enzyme in the presence of various effectors. Control experiments were performed with another modified form of the enzyme, pyridoxal phosphorylase. It is suggested that the transition-state analogue pyridoxal pyrophosphate, bound at the active site, mimics the actual configuration of enzyme plus substrate achieved during the normal catalytic reaction and therefore traps the enzyme in an activated conformation. These findings agree well with recent results obtained with the alternate transition-state analogue pyridoxal pyrophosphate glucose [Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S., & Fukui, T. (1981) *J. Biol. Chem. 256*, 10759] and therefore provide further evidence for the "interacting phosphates" hypothesis presented in the latter paper.

The focal point of most studies on the catalytic mechanism of glycogen phosphorylase in recent years has been the role of the coenzyme pyridoxal phosphate. The phosphate moiety has been shown to be the catalytically essential component of the coenzyme by a variety of studies involving analogue replacement (Kastenschmidt et al., 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972; Feldmann et al., 1972, 1974; Vidgoff et al., 1974; Feldmann & Helmreich, 1976; Parrish et al., 1977; Shimomura & Fukui, 1978; Hoerl et al., 1979; Chang & Graves, 1982). The phosphate moiety has been studied quite

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extensively by ^{31}P nuclear magnetic resonance (NMR)[1] (Feldmann & Hull, 1977; Hoerl et al., 1979; Withers et al., 1979, 1981a) and conflicting results obtained on its protonation state during catalysis. The structure of the enzyme deduced from X-ray crystallographic studies shows the pyridoxal phosphate to be located in the active site (Sygusch et al., 1977) with the coenzyme oriented such that its aromatic ring is embedded in a pocket in the C-terminal domain, with the phosphate moiety in a cleft between the C- and N-terminal domains, pointing toward the substrate binding site. Similar observations were made with phosphorylase *b* (Johnson et al., 1980). The phosphate moiety of the coenzyme is therefore the functional group situated nearest to the substrate. The crystal structure of phosphorylase *a* was, however, performed on a glucose-inhibited form of the enzyme, but results of recent studies using glucose cyclic 1,2-phosphate to replace glucose in a difference-Fourier analysis (Withers et al., 1982) show that the two glucose moieties bind at essentially the same site and that the phosphate of the substrate analogue is oriented toward the phosphate of the coenzyme. However, a distance of some 6.8 Å (P to P) separates the two. The crystal form obtained by such a soaking experiment is not, however, a fully activated form, since it is known that if the crystal is soaked with both glucose cyclic 1,2-phosphate and maltopentaose a far greater structural reorganization is observed, producing data which are uninterpretable.

Various solution studies have suggested that the two phosphate groups are held close together in the fully activated enzyme. The first intimations of this possibility came from the observation of potent inhibition of phosphite-activated PL-phosphorylase *b* by pyrophosphate. This inhibitor showed competitive kinetics against both substrate glucose-1-P and activator phosphite and a stoichiometry of binding of one pyrophosphate per monomer, suggesting that it was able to bind to the two subsites simultaneously (Parrish et al., 1977). Further evidence for this came from the rapid rate of reconstitution of apophosphorylase by pyridoxal pyrophosphate (Shimomura et al., 1978). This has been substantiated by more recent studies (Withers et al., 1982) showing that PL-phosphorylase *b* is more potently inhibited by methylene diphosphonate (P–P separation = 3 Å) than by propylene diphosphonate (P–P separation = 6 Å). Further support comes from the recent observation of catalytically relevant cleavage of the coenzyme–substrate adduct PLPP–α-glucose bound to phosphorylase, in the presence of oligosaccharide, resulting in transfer of the glucose moiety to the oligosaccharide (Withers et al., 1981b; Takagi et al., 1982). In the latter example, the two phosphates are joined via a pyrophosphate linkage, thus representing an analogue of the transition state of the proposed reaction pathway.

This paper describes studies performed on PLPP-phosphorylase *b* and PL-phosphorylase *b* by means of ^{31}P NMR techniques to obtain nucleotide binding constants, and by ultracentrifugation studies to obtain quaternary structural information. ^{31}P NMR has been used previously (Withers et al., 1979, 1981a) to obtain data on the affinity of phosphorylase *b* for nucleotide under various conditions. By means of a computer-assisted analysis of the line shape obtained for free and bound AMPS, it is possible to extract values for both the dissociation constant, K_D, and the off rate constant, k_{-1}, of the nucleotide for each condition studied. The nucleotide binding constant can be used as an indicator of the enzyme conformation, since activated R-state phosphorylase exhibits a high affinity for nucleotide and inhibited T-state phosphorylase exhibits a low affinity. Ultracentrifugation studies have also been used as a probe of the "activation state" of the enzyme (Withers et al., 1979). It has been shown previously (Kastenschmidt et al., 1968; Wang et al., 1970) that phosphorylase *b* exists as a dimer in the absence of ligands but associates to a tetramer on binding AMP. Glucose, however, can reverse this nucleotide-induced association and force it back to a dimer, as can caffeine (Wang et al., 1965; Withers et al., 1979). Addition of maltoheptaose, an oligosaccharide analogue of glycogen, to phosphorylase *b*, even in the presence of substrates and activators (Wang et al., 1970), causes dissociation to a dimeric form, but this is an "active" R-state dimer and appears to be of different monomer conformation from that induced by the T-state effectors. Therefore, R-state phosphorylase can exist as a tetramer or active dimer, while T-state phosphorylase always exists as the inactive dimer.

The results described in this paper using ^{31}P NMR and ultracentrifugation techniques all provide evidence for the "interacting phosphates" hypothesis previously proposed.

Materials and Methods

All buffer chemicals and substrates were obtained from Sigma Chemical Co. except for DTT which was obtained from Bio-Rad Laboratories. AMPS was obtained from Boehringer-Mannheim, and D_2O was from Bio-Rad Laboratories. Maltopentaose, PLPP, and PLPP-α-Glc were kind gifts of Dr. T. Fukui. Potassium phosphite was from ICN Pharmaceuticals. Rabbit muscle phosphorylase *b* was prepared, resolved, and reconstituted as described previously (Withers et al., 1981a, 1982). Reconstitutions were performed with the following molar excesses of PLP and analogues: pyridoxal, 50-fold; PLPP and PLPP-α-Glc, 2-fold. Apoenzyme routinely exhibited less than 0.1% activity and could be reconstituted with PLP to normal activity.

^{31}P NMR spectra were recorded at 109.29 MHz on a Bruker HX270 superconducting spectrometer operating in the Fourier transform mode with quadrature phase detection, at 30 °C. A spectral width of ±5000 Hz was generally employed with a 50–70° pulse angle (15–20 μs) and a repetition time of 1.6 s. Exponential line broadening used prior to Fourier transformation was generally 20 Hz, and all line-width data have been corrected for this.

Sample size was usually 1.2 mL in a flat-bottomed 10-mm tube, with enzyme concentrations between 0.75 and 1.1 mM calculated for the phosphorylase *b* monomer of molecular weight 97 333. Enzyme was concentrated by means of a Millipore immersible concentrator, and solutions of effectors, dissolved in D_2O NMR buffer at pH 6.8, were added directly to the NMR tube as required. The buffer used in all NMR experiments was 50 mM triethanolamine hydrochloride, 100 mM KCl, 1 mM EDTA, and 1 mM DTT (pH 6.8 meter reading) made up in D_2O previously treated with Chelex to remove any paramagnetic impurities. The D_2O present in the buffer was used for field/frequency lock, and a 1-mm tube containing 85% phosphoric acid was inserted for chemical shift referencing. Computer-assisted line-shape analysis of the binding of AMPS was performed as described previously (Withers et al., 1979). T_1's were measured by the progressive saturation method (Freeman & Hill, 1971).

The ultracentrifugation experiments were performed on a Spinco Model E analytical ultracentrifuge at a rotor speed of 56 000 rpm and a temperature of 20 ± 0.1 °C. Sedimentation

[1] Abbreviations: NMR, nuclear magnetic resonance; PL, pyridoxal; PLPP, pyridoxal 5′-pyrophosphate; PLPP-α-Glc, pyridoxal(5′)diphospho(1)-α-D-glucose; DTT, dithiothreitol; AMPS, adenosine 5′-*O*-thiomonophosphate; PLFP, pyridoxal 5′-phosphofluoridate; EDTA, ethylenediaminetetraacetic acid.

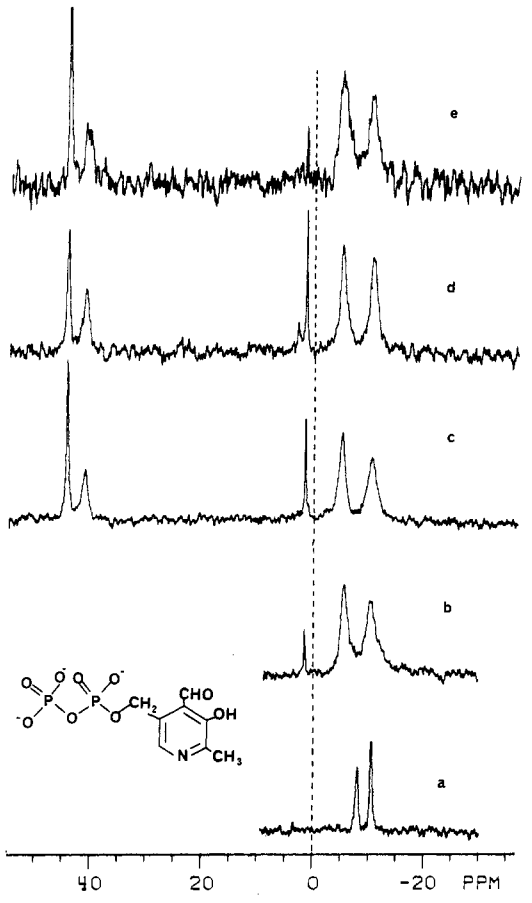

FIGURE 1: ^{31}P NMR spectra of PLPP and PLPP-phosphorylase in the presence of various effectors. (a) PLPP, pH 6.8; (b) PLPP-phosphorylase *b* (1.14 mM) (0–14 h); (c) PLPP-phosphorylase *b* (1.11 mM) + AMPS (2.18 mM) (14–28 h); (d) PLPP-phosphorylase *b* (0.97 mM) + AMPS (1.76 mM) + glucose (100 mM) + caffeine (3 mM) (37–50 h); (e) PLPP-phosphorylase *b* (0.91 mM) + AMPS (1.8 mM) + maltopentaose (55 mM) (24–34 h). Inset: Structure of PLPP.

coefficients determined from schlieren patterns were corrected for the viscosity and density of the buffer to water at 20 °C. Viscosities were determined by means of a Cannon-Manning semimicro viscometer at 20 °C. Protein concentration was 4–5 mg/mL, and the buffer used was the same triethanolamine buffer as used for the NMR experiments. Activity measurements were performed as described previously (Engers et al., 1970).

Results

^{31}P NMR Studies. (A) PLPP-phosphorylase b. The ^{31}P NMR spectrum of PLPP free in solution at pH 6.8 is shown in Figure 1a. The resonances at δ −10.2 and −7.6 are assigned to the α- and β-phosphates, respectively, of PLPP by comparison with the spectrum of adenosine diphosphate and on the basis of the pH dependences of their chemical shifts (Figure 2). Figure 1b shows the corresponding spectrum of PLPP-phosphorylase *b* at pH 6.8. The two peaks seen at δ −5.4 ($\Delta\nu$ = 140 Hz) and δ −10.2 ($\Delta\nu$ = 210 Hz) are assigned to the β and α peaks, respectively, of PLPP bound at the active site by comparison with Figure 1a; the small peak at δ +1.8 is due to inorganic phosphate. The α-phosphate resonance is not shifted significantly upon binding, but the β-phosphate resonance is shifted 2.2 ppm downfield. This may imply some deprotonation upon binding, mostly likely due to the presence of positively charged counterionic groups forming its binding pocket within the active site. Interpolation of the chemical

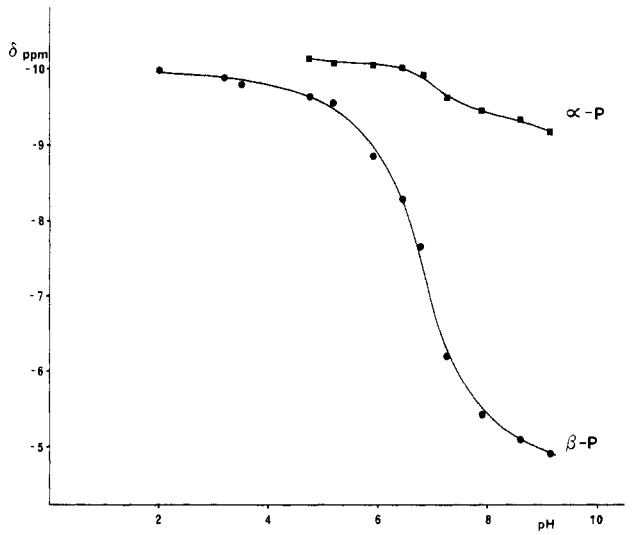

FIGURE 2: pH dependence of the chemical shifts of the α- and β-phosphates of PLPP.

shift for enzyme-bound PLPP onto the titration curve obtained for the chemical shifts of the α- and β-phosphates of PLPP free in solution (Figure 2) indicates that the environment of the bound β-phosphate is equivalent to that of the free PLPP at pH 8.2. Such a downfield shift has been observed for many phosphate esters upon binding to their respective enzymic sites.[2]

Upon addition of nucleotide activator (AMPS) to the sample, two major changes are observed to occur in this region of the spectrum (Figure 1c). First, the inorganic phosphate peak has increased markedly, and second, the resonance due to the terminal (β) phosphate has again shifted downfield a further 0.26 ppm. The increase in the inorganic phosphate peak is presumably due to slow hydrolysis of the terminal phosphate of PLPP, leaving PLP in the active site and releasing P_i. This slow hydrolysis is accompanied by a small increase in catalytic activity, as would be expected.

The downfield shift of the β-PLPP resonance upon nucleotide activation of the enzyme is presumably due to a further deprotonation of the phosphate moiety. It now resonates at a position coincident with that of free PLPP at pH 9 or higher (Figure 2), i.e., the fully deprotonated species. The nucleotide activation has caused a lowering of the pK_a of this terminal phosphate, assuming that this shift does indeed represent deprotonation. This would most easily be explained by the creation of a more positively charged environment for the phosphate. Such movement of basic amino acids upon activation to create a phosphate binding site is quite reasonable and has in fact been demonstrated by recent X-ray crystallographic studies (Withers et al., 1982). The shift in the ^{31}P NMR resonance of the β-phosphate of PLPP free in solution upon deprotonation is accompanied by a small downfield shift in the α-phosphate resonance (Figure 2). Such a downfield-shifted position of the α-phosphate resonance is not, however, observed for PLPP-phosphorylase *b* upon activation. In fact, it still resonates at a position equivalent to that observed for free PLPP at pH 6.8. If a deprotonation of the terminal phosphate has indeed occurred, then this must be accompanied

[2] A reviewer has pointed out the possibility that the opposite peak assignment is the correct one, with the free α resonance at −10.2 ppm shifting to −5.4 ppm upon binding and the free β resonance at −7.6 ppm shifting to −10.2 ppm upon binding. We consider this less likely since the assigned resonance of the β-phosphate shows more sensitivity to ligand binding, as would be expected for an ionizable phosphate. An error in assignment would not negate the major thrust of our argument.

Table I: Off-Rate (k_{-1}) and Dissociation (K_D) Constants for AMPS Binding Calculated from NMR Data[a]

system	k_{-1} (s^{-1})	K_D (μM)
PLPP-phos *b* + AMPS	40	40
PLPP-phos *b* + AMPS + Glc	60	40
PLPP-phos *b* + AMPS + Glc + caffeine	80	40
PLPP-phos *b* + AMPS + maltopentaose	45	40
PL-phos *b* + AMPS	45	20
PL-phos *b* + AMPS + Glc	60	20
PL-phos *b* AMPS + Glc + phosphite	182	100
PL-phos *b* + AMPS + Glc + phosphite + caffeine	1000	300
PL-phos *b* + AMPS + pyrophosphate	38	20
PL-phos *b* + AMPS + pyrophosphate + caffeine	40	20
PL-phos *b* + AMPS + pyrophosphate + caffeine + Glc + phosphite	600	200
PLFP-phos *b* + AMPS	55	50
phos *b* + AMPS[b,c]	200	200
phos *b* + AMPS + Glc[c]	800	300
phos *b* + AMPS + Glc-1-P[b]	150	150
thiophosphorylase *a* + AMPS[b]	25	2

[a] Concentration of enzyme and ligands are described in Figures 1-3 and text. phos *b* = phosphorylase *b*. [b] Taken from Withers et al. (1981a). [c] Taken from Withers et al. (1979).

by an effective upfield shift of the α-phosphate resonance. Whatever the cause of this shift, be it a local electronic effect or a distortion of the phosphate, it is reminiscent of the upfield shift observed for the phosphate of PLP in native enzyme upon "activation" by both nucleotide and glucose cyclic 1,2-phosphate (Withers et al., 1981a). This may prove to be significant since it is the α-phosphate of PLPP which is sterically equivalent to the phosphate of PLP in native phosphorylase. However, in the case of PLPP-phosphorylase *b*, this upfield shift cannot be the result of protonation of the phosphate and may well be a result of distortion.

The two downfield peaks at δ 44.3 and 41.1 (Figure 1c) are assigned to the free and bound AMPS, respectively. The observation of such sharp distinct resonances indicates that the nucleotide is indeed bound tightly to this form of the enzyme. Values for the off-rate (k_{-1}) and dissociation constants (K_D) for AMPS binding to the enzyme, along with some similar constants for native phosphorylases *a* and *b* published previously, are presented in Table I. In this type of analysis, the off-rate constant is the more reliable parameter, and it is clear from these data that the binding of nucleotide to PLPP-phosphorylase *b* is extremely tight, resembling phosphorylase *a* more than phosphorylase *b* in this parameter. The enzyme therefore exists in an R conformation.

Addition of 100 mM glucose to this nucleotide-activated enzyme sample resulted in no significant changes to the ^{31}P NMR spectrum (not shown). The AMPS is still bound very tightly (see Table I) ($k_{-1} = 60\ s^{-1}$), and there has been no shift of the resonances due to PLPP. Subsequent addition of 3 mM caffeine to this sample (Figure 1d) also had no significant effect upon the spectrum. The inorganic phosphate peak (δ −1.8) has increased as expected, and the small peak at δ 3.1 is probably due to a very small amount of AMP formed as a decomposition product of AMPS in this lengthy experiment. A small increase in the off-rate constant (k_{-1}) is observed, but this is insignificant by comparison with the changes observed for native phosphorylase *b* under these circumstances (Table I). The inability of the two T-state inhibitors, glucose and caffeine, both present at concentrations 50–100 times greater than their dissociation constants with native phosphorylase, to either significantly weaken the binding of AMPS or shift the resonances of PLPP back to the positions observed in unliganded PLPP-phosphorylase *b* implies that the PLPP-phosphorylase *b*–nucleotide complex is locked into an activated R conformation. Furthermore, addition of the oligosaccharide maltopentaose (55 mM) to the nucleotide-activated PLPP-phosphorylase *b* had no significant effect on the spectrum (Figure 1e).

One apparent problem with all the spectra of PLPP-phosphorylase *b* is that the resonances due to the α- and β-phosphates of PLPP are each of significantly greater area than that of the bound AMPS. Since all these species would be expected to be present in equal proportion, the most likely explanation for such inequivalence in signal magnitude would be differences in spin–lattice relaxation times (T_1) for these nuclei. T_1 values for the α- and β-phosphates of PLPP and for free and bound AMPS were determined as described under Materials and Methods, and values of 6.0 s for both free and bound AMPS and of 2.7 and 2.5 s for the α- and β-phosphates of bound PLPP, respectively, were obtained. This difference in T_1 values, with the 1.6-s pulse delay used in the experiment, adequately accounts for the observed discrepancies in signal magnitude.

(*B*) *PL-phosphorylase b.* Since other modified phosphorylases with altered pyridoxal phosphate moieties have been shown to bind AMP tightly (Kastenschmidt et al., 1968), it was thought to be important to investigate one such modified enzyme by similar techniques as a control of our experiments with PLPP-phosphorylase. PL-phosphorylase was selected, as it has been shown previously to exhibit high affinity for nucleotide (Kastenschmidt et al., 1968), since considerable kinetic analysis has been applied to it (Parrish et al., 1977; Withers et al., 1982) and since it also provides the opportunity, in the presence of pyrophosphate, to study a noncovalent analogue of PLPP-phosphorylase *b*.

The spectrum of PL-phosphorylase with AMPS added is shown in Figure 3a. The only region displayed is that encompassing the resonances of free and bound AMPS. As can be seen, the nucleotide is tightly bound, and this is reflected in the off-rate and dissociation constants (Table I). Upon addition of glucose (52 mM) (Figure 3b), very little difference is observed, and very similar off-rate and dissociation constants are determined. However, upon subsequent addition of potassium phosphite (75 mM) to this sample (Figure 3c), a very distinct weakening of binding is shown by the broadening of the lines, and this is reflected in the increase in off-rate and dissociation constants. Phosphite has been suggested previously to bind to pyridoxal phosphorylase in the site normally occupied by the phosphate of PLP (Parrish et al., 1977), and it has recently been predicted on the basis of kinetic studies (Withers et al., 1982) that the presence of phosphite or a suitable analogue is essential for formation of the glucose binding site. This latest evidence provides further proof that phosphite must be present for glucose to bind. Finally, addition of caffeine (3.5 mM) to this same sample results in extreme weakening of the nucleotide binding as shown by the single exchange-averaged signal for both free and bound AMPS (Figure 3d). This is reflected in a large increase in the off-rate constant (Table I). It therefore appears that even though pyridoxal phosphorylase exists in slightly more of an R conformation than native phosphorylase, it can quite easily be pushed into a T conformation by T-state effectors.

A similar series of experiments was carried out with pyridoxal phosphorylase in the presence of pyrophosphate (spectra not shown). The same general observations were made as in the absence of pyrophosphate, except that the pyrophosphate

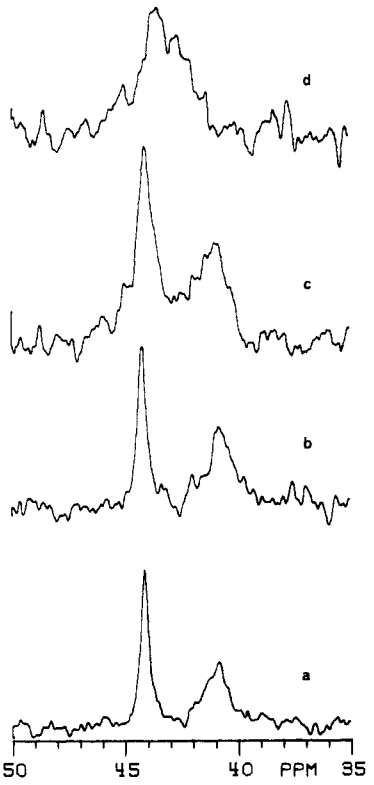

FIGURE 3: AMPS region of ^{31}P NMR spectra of PL-phosphorylase *b* in the presence of various effectors. (a) PL-phosphorylase *b* (0.93 mM) + AMPS (1.68 mM); (b) PL-phosphorylase *b* (0.92 mM) + AMPS (1.73 mM) + glucose (52 mM); (c) PL-phosphorylase *b* (0.91 mM) + AMPS (1.71 mM) + glucose (52 mM) + phosphite (7.5 mM); (d) PL-phosphorylase *b* (0.88 mM) + glucose (49 mM) + phosphite (7 mM) + caffeine (3.5 mM).

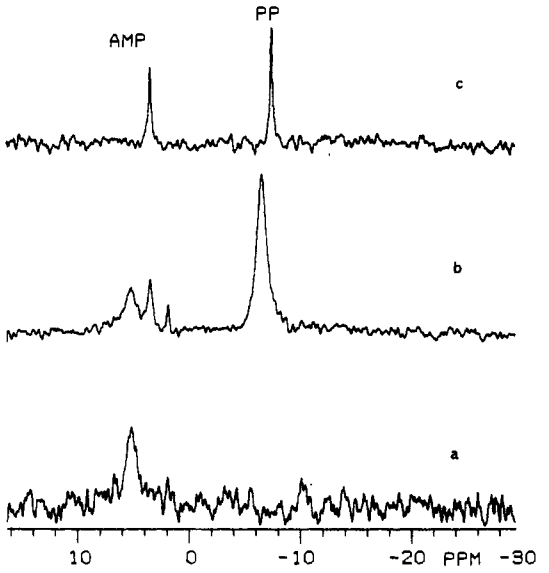

FIGURE 4: ^{31}P NMR spectra of AMP and pyrophosphate in the presence and absence of PL-phosphorylase *b*. (a) PL-phosphorylase *b* (0.9 mM) + AMP (0.75 mM); (b) PL-phosphorylase *b* (0.9 mM) + AMP (1.4 mM) + pyrophosphate (1.2 mM); (c) AMP and pyrophosphate, pH 6.8.

appeared to help stabilize the R conformation a little, as reflected in the smaller effect of the T-state ligands on the off-rate constant (Table I). This pyrophosphate moiety, bound at the active site, therefore has the effect of stabilizing an activated conformation of the enzyme; however, when it is noncovalently bound, it can be "competed out" by high concentrations of T-state effectors and the enzyme converted to a T-state conformation. When covalently bound, this is not possible, and the enzyme is permanently locked into an R conformation. The weakening of binding of pyrophosphate is seen on careful examination of the pyrophosphate resonance, which in the presence of nucleotide-activated PL-phosphorylase *b* is broadened ($\Delta\nu$ = 120 Hz) and shifted 0.36 ppm downfield from its position free in solution (see Figure 4 for a similar example). Upon addition of the two T-state effectors plus phosphite, the resonance is shifted 0.20 ppm back upfield toward the resonance for free pyrophosphate, and it is narrowed considerably ($\Delta\nu$ = 10 Hz) (not shown). This indicates a dramatic weakening of pyrophosphate binding concomitant with the 15-fold weakening of AMPS binding (Table I). This investigation was further extended by means of a line-shape analysis of the pyrophosphate resonance. In order to perform as simple an analysis as possible, it was assumed that even if different binding modes for the two phosphate moieties existed, the exchange between them was sufficiently rapid to allow a two-site exchange approximation (free and averaged bound pyrophosphate) to be employed. The best fit was obtained if it was assumed that the bound pyrophosphate resonated with a chemical shift equivalent to the average of the shifts for trianionic and tetraanionic pyrophosphate. Values obtained in this way for the binding of pyrophosphate to nucleotide-activated PL-phosphorylase *b* were K_D = 30 μM and k_{-1} = 275 s^{-1}. These are reasonable values, when compared to the value for K_D = 80 μM determined for the binding of pyrophosphate to PL-phosphorylase *b* in the absence of nucleotide (Parrish et al., 1977). In the presence of the two T-state effectors plus phosphite, the constants increased greatly (K_D = 5.5 mM, k_{-1} = 6500 s^{-1}), as would be expected for such an R-state promoter bound to a T-state enzyme.

(C) Binding of AMP and Pyrophosphate to PL-phosphorylase b. Pyridoxal phosphorylase *b* provides an ideal system for studying the binding of AMP to phosphorylase by ^{31}P NMR since it binds AMP very efficiently and contains no phosphate group whose resonance could mask those of the free and bound AMP.

Figure 4a shows a spectrum of pyridoxal phosphorylase (0.9 mM) plus AMP (0.75 mM). The only resonance observed is that of bound AMP, δ 5.1, with a line width of 80 Hz. This indicates that all the AMP is bound and that it is bound quite tightly since there is very little exchange broadening. (Natural line width for both PLP and bound AMPS with native phosphorylase = 70 Hz.) Addition of excess AMP (total AMP concentration = 1.4 mM) and also pyrophosphate (1.2 mM) gives the spectrum shown in Figure 4b. Here, one can easily distinguish bound and free AMP (δ 5.1 and 3.4, respectively), a small contaminant of inorganic phosphate (δ 1.8), and a strong signal from pyrophosphate (δ −6.5), as observed for AMPS-activated PL-phosphorylase *b*. Figure 4c shows the ^{31}P NMR spectra of free AMP and pyrophosphate for reference. A line-shape analysis was applied to the AMP region of Figure 4b, and binding parameters of k_{-1} = 40 s^{-1} and K_D = 50 μM were obtained. These compare favorably with the previous literature value (Kastenschmidt et al., 1968) of K_D = 53 μM for AMP binding to PL-phosphorylase *b*.

The resonances for free and bound AMP are interesting in that the bound AMP resonates 1.7 ppm downfield of free AMP at pH 6,8, whereas bound AMPS resonates 3.2 ppm upfield of free AMPS. This may imply that the chemical shift position for such bound resonances is dominated by the ionization state of the phosphate or thiophosphate, since it has been shown previously (Jaffe & Cohn, 1978) that fully deprotonated AMPS resonates upfield of AMPS at neutral pH, whereas fully deprotonated AMP resonates downfield of AMP

Table II: Sedimentation Constants of Modified Phosphorylases[a]

system	$s_{20,w}$ (S)
PLPP-phos *b*	8.7
PLPP-phos *b* + AMP (1)	13.4
PLPP-phos *b* + AMP (1) + Glc (100)	12.7
PLPP-phos *b* + AMP (1) + Glc (100) + caffeine (4)	12.5
PLPP-phos *b* + Glc (100)	8.3
PLPP-phos *b* + caffeine (4)	8.1
PLPP-phos *b* + AMP (1) + MPose (5)	12.6
PLPP-phos *b* + AMP (1) + MPose (35)	10.1
PLPP-phos *b* + AMP (1) + MPose (70)	8.5
PL-phos *b*	8.5
PL-phos *b* + AMP (1)	13.2
PL-phos *b* + AMP (1) + phosphite (10)	13.3
PL-phos *b* + AMP (1) + Glc (50)	12.5
PL-phos *b* + AMP (1) + Glc (50) + phosphite (10)	8.3
PL-phos *b* + AMP (1) + MPose (50)	11.5
PL-phos *b* + AMP (1) + MPose (50) + pyrophosphate (1)	8.6
PL-phos *b* + AMP (1) + pyrophosphate (1)	12.7
PLPP-α-Glc-phos *b*	8.7
PLPP-α-Glc-phos *b* + AMP (1)	12.8

[a] Numbers in parentheses refer to concentrations (in millimolar units). MPose = maltopentaose; phos *b* = phosphorylase *b*.

at neutrality. This is also in accord with recent chemical evidence (Withers & Madsen, 1980) that AMP binds to phosphorylase in a dianionic form.

Ultracentrifugal Studies. Results of ultracentrifugation studies with phosphorylase reconstituted with PLPP, PLPP-α-Glc, and PL in the presence of various ligands are presented in Table II. All $s_{20,w}$ values presented were derived from sedimentation velocity determinations and have been corrected for the viscosity of the buffer solution in each case. These corrections were quite significant when the buffer contained high concentrations of maltopentaose.

(*A*) *PLPP-phosphorylase b.* Unliganded PLPP-phosphorylase *b* is shown to exist as a dimer (8.7 S) under these conditions (Table II) just as does native phosphorylase *b*. Addition of 1 mM nucleotide (AMP or AMPS) leads to complete conversion to a tetrameric form (13.4 S), exactly as in the case of native phosphorylase *b*. Subsequent addition of glucose (100 mM) and additional caffeine (4 mM) fails to cause dissociation of the tetramer to dimer, however, in contrast to the situation obtained with native phosphorylase *b* when the enzyme would have been totally dissociated to dimer in the presence of equivalent concentrations of either one of these T-state effectors. Indeed, an attempt was made to remove bound nucleotide from AMP-treated PLPP-phosphorylase *b* by prolonged dialysis against a suspension of charcoal in a buffer containing 50 mM glucose, as described previously (Withers et al., 1979). Enzyme treated in this way still sedimented as a tetramer in the presence of 100 mM glucose, but some dimer formation was evident when 10 mM caffeine was added to this same sample. However, when PLPP-phosphorylase which had never been exposed to nucleotide was investigated in the ultracentrifuge in the presence of glucose or caffeine, it did sediment as a dimer, in the same way as unliganded enzyme. These experiments again indicate that nucleotide-activated PLPP-phosphorylase *b* is locked into an activated R state.

Addition of the glycogen analogue maltopentaose to nucleotide-activated PLPP-phosphorylase *b* results in dimer formation when the concentration of maltopentaose is sufficiently high. It was found that a concentration of 70 mM maltopentaose was necessary to force the enzyme completely into the active dimeric form. At lower concentrations of maltopentaose, a single broad peak with a sedimentation constant between that of dimer and tetramer was observed. This single peak represents an average of the tetramer and dimer sedimentation constants weighted by the relative concentrations of each species present. Observation of a single average peak rather than two distinct peaks is evidence that the interconversion rate between the tetramer and the active dimer is fast on the time scale of the sedimentation. The interconversion between tetramer and T-state dimer is, however, slow since separate peaks are observed in this case (Schachmann, 1959). The fast interconversion situation has been observed previously for the binding of maltoheptaose to native phosphorylase *a* (Metzger et al., 1967). This difference in interconversion rates, in conjunction with the fact that the enzyme shows a high affinity for nucleotide in the presence of oligosaccharide, is good evidence that the dimer observed under these conditions is an active R-state dimer and not the inhibited T-state dimer. PLPP-phosphorylase *b* therefore appears to behave like native phosphorylase *b* in the presence of its phosphorylated substrate in terms of its activated R-state conformations, but unlike phosphorylase *b* in terms of conversion to a T state.

Similar observations have been made in ultracentrifugal studies with the alternate transition-state complex PLPP-α-Glc-phosphorylase *b* (Table II). This too sediments as a dimer in the absence of ligands but forms a tetramer in the presence of nucleotide activators. Addition of maltopentaose, however, causes immediate formation of PLPP-phosphorylase *b*, so no information can be obtained on effects of this ligand.

(*B*) *PL-phosphorylase b.* PL-phosphorylase *b* was shown to exist as a dimer (8.5 S) in the absence of ligands (Table II) but again was converted to tetramer upon nucleotide activation. Subsequent addition of phosphite (10 mM) had no further effect on its oligomeric state.

Addition of glucose (50 mM) to AMP-activated PL-phosphorylase *b* was insufficient to force the enzyme back to a dimer. However, subsequent addition of phosphite to this sample forced the enzyme into a dimeric form (8.3 S) (Table II), providing further proof that glucose is unable to bind (or at least binds very poorly) in the absence of phosphite. PL-phosphorylase, even when activated by nucleotide, is therefore able to revert to its inactive T form in contrast to PLPP-phosphorylase *b*, which cannot. Effects of oligosaccharide on this form of the enzyme are also interesting. Addition of maltopentaose (50 mM) to the nucleotide-activated enzyme is insufficient to force it into a dimeric form, since the species observed (11.5 S) is mainly tetrameric. Under these conditions, PLPP-phosphorylase was totally in the active dimer form. However, addition of pyrophosphate (1 mM) to this complex resulted in complete conversion to the active dimer (8.6 S) since the system is now acting as a noncovalent analogue of the transition-state complex. Addition of pyrophosphate to nucleotide-activated PL-phosphorylase *b* in the absence of oligosaccharide, however, resulted in stabilization of the tetrameric form (12.7 S). There is therefore positive allosteric interaction between the binding of pyrophosphate at the active site and of oligosaccharide.

PL-phosphorylase *b*, therefore, is fully capable of conversion into a T form and only acts like a true R-state enzyme in the presence of pyrophosphate, and then in a reversible manner.

Discussion

Both the results from the ^{31}P NMR studies on the tertiary structure of the enzyme and those from ultracentrifugal experiments on its quaternary organization point to the same conclusion. When phosphorylase *b* is reconstituted with the putative transition-state analogue PLPP, the enzyme now

mimics the behavior of native phosphorylase in the presence of its phosphorylated substrate. Further, when AMP is added as an activator, the structure is locked into a R conformation. It appears, on the basis of both sets of results, that PLPP-phosphorylase *b* behaves in the same manner as native phosphorylase *b* in the presence of substrate in all its activated, "R"-state conformations but that it is unable to adopt an inactive T-state conformation in response to T-state inhibitors, presumably due to the covalently bound substrate analogue. Experiments performed with PL-phosphorylase *b* serve as a control for this hypothesis since it has been shown previously (Kastenschmidt et al., 1968; Parrish et al., 1977) that this is also a modified phosphorylase *b* which can bind nucleotide very effectively. However, results presented here demonstrate that a major difference exists between PLPP-phosphorylase *b* and PL-phosphorylase *b* in that the latter is fully capable of reverting to the T state, even in the presence of nucleotide. On addition of pyrophosphate, PL-phosphorylase shows some of the characteristics of PLPP-phosphorylase since it is able to mimic the same structure, but in a noncovalent fashion. However, even in the presence of pyrophosphate, it cannot be locked into an R-state conformation since it is always possible to displace the pyrophosphate with a sufficient concentration of T-state effector. Further evidence that PLPP is capable of inducing an activated R-state conformation in phosphorylase comes from some recent work (Fukui et al., 1982) on potato phosphorylase. They studied the oligosaccharide binding affinity of potato phosphorylase reconstituted with various pyridoxal phosphate analogues. Since the potato enzyme contains no glycogen storage site external to the active site, this system provides a better measure of the affinity at the active site for oligosaccharide. They found that PLPP-phosphorylase *b* had the greatest affinity for oligosaccharide of all the analogues tested and that the affinity for amylopectin was 5 times greater with PLPP-phosphorylase than with native phosphorylase. This indicates an active R-state conformation of the active site.

The finding that PLPP-phosphorylase *b* can easily be locked into an R-state conformation provides further evidence for the theory recently proposed (Withers et al., 1981b) and developed further (Withers et al., 1982) that the phosphates of the substrate and pyridoxal phosphate actually interact during the normal catalytic reaction and that this interaction provides some source of catalytic enhancement of the enzyme's activity, possibly involving the coenzyme phosphate as an electrophile. It is therefore gratifying to note that phosphorylase *b* reconstituted with the alternate transition-state analogue PPLP-α-glucose shows a similar response to activation to that shown by PLPP-phosphorylase *b* in terms of its quaternary structure and has a similar affinity for nucleotide [$k_{-1} = 36\ s^{-1}$ and $K_D = 30\ \mu M$ for AMPS binding (Withers et al., 1981a)].

In addition to its role in catalysis, the PLP phosphate may be important as a trigger of conformational changes throughout the molecule, particularly if the T $\leftrightarrow$ R conformation changes. Some of the data in this paper could be interpreted in this manner, but the possibility remains speculative until more direct evidence can be obtained.

Finally, it seemed that as nucleotide-activated PLPP-phosphorylase *b* appeared to be locked into an activated R-state conformation, this might provide an ideal system for crystallization since complete stabilization into one conformer appears to be essential for success in growing crystals for X-ray crystallographic analysis. The structure of an activated form of phosphorylase would obviously be of great interest. We have indeed succeeded in growing good crystals of PLPP-phosphorylase *b*, of space group $P2_12_12$, in the presence of both AMP and maltopentaose. These crystals diffract well and are presently undergoing a full X-ray crystallographic analysis.

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