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Ionization of Pyridoxal 5′-Phosphate and the Interactions of AMP-S and Thiophosphoseryl Residues in Native and Succinylated Rabbit Muscle Glycogen Phosphorylase *b* and *a* as Inferred from ^{31}P NMR Spectra†

Maximilian Hoerl, Knut Feldmann, Klaus D. Schnackerz, and Ernst J. M. Helmreich*

ABSTRACT: Rabbit skeletal muscle phosphorylase *b* or *a* dissociates on treatment with succinic anhydride, yielding a mixture of about 40% monomer and 60% dimer with a residual activity of about 15%. The phosphate signal of pyridoxal phosphate in the native, inactive form of phosphorylase *b* in the absence of ligands was assigned to a shielded protonated form. The same resonance line with the identical chemical shift was observed in a monomer–dimer mixture of succinylated phosphorylase *b*, indicating that dissociation had no effect on the ionization of the phosphate group of pyridoxal phosphate. This is not compatible with a location of the cofactor at or near the subunit interface. Glucose, an inhibitory substrate analogue, binds at the α-glucose 1-phosphate site. Experiments with pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a* have shown that the 5′ side chain of the cofactor analogue reports the binding of glucose. These findings were interpreted in support of a pyridoxal phosphate location near the catalytic site. Binding of the allosteric effector adenosine 5′-*O*-thiomonophosphate (AMP-S) to succinylated phosphorylase *b* caused the protonated form of the cofactor to decline in favor of the dianionic form, the amount of which correlates with the dimer concentration. In native phosphorylase *a*, the phosphate of pyridoxal phosphate is in the dianionic form, whereas the main phosphate signal in succinylated phosphorylase *a* represents the protonated form. Addition of AMP-S to the latter leads to an increase in the dianionic form, but a complete conversion was not achieved. At pH 6.5, the optimum for activity, the dianionic thiophosphoseryl residue of native phosphorylase *a* forms a stable salt bridge. At more alkaline pH (7.7), attachment of the thiophosphoseryl residue is partly abolished, but this residue can be reattached on adding AMP-S, indicating that the phosphates of the nucleotide and of the phosphoseryl residue bind to different but cooperative sites. The succinylated AMP-S phosphorylase *a* complex has a much shorter lifetime even at a more acid pH (7.35) than the corresponding complex with native phosphorylase *a* at pH 7.67, leaving most of the thiophosphoseryl side chains mobile. This might be related to the much greater dependence of succinylated phosphorylase *a* for adenosine 5′-monophosphate to express activity.

The complete sequence analysis of the 841 amino acids forming a monomer of rabbit skeletal muscle glycogen phosphorylase *b* (EC 2.4.1.1) by Titani et al. (1977) and the refinement of the X-ray diffraction patterns of phosphorylase *a* (Sygusch et al., 1977; Kasvinsky et al., 1978 a,b) and of phosphorylase *b* (Weber et al., 1978) to a resolution of 3.0 Å impressively attest the progress made in the elucidation of the structure of this important regulatory enzyme. These advances should help to understand the role of pyridoxal-P[1] which is essential for the activity of all known α-glucan phosphorylases. Studies in the last decade have provided evidence—although none of it conclusive, but, when taken as a whole, nevertheless quite impressive—that among all the functional groups of pyridoxal-P the 5′-phosphate group is the most attractive candidate for a role in catalysis (Kastenschmidt et al., 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972; Feldmann et al., 1972, 1974, 1978; Vidgoff et al., 1974; Feldmann & Helmreich, 1976; Parrish et al., 1977; Feldmann & Hull, 1977; for reviews, see Fischer et al., 1970; Graves & Wang, 1972). To advance the knowledge on the structure of this enzyme in solution and specifically to study the ionization of the 5′-phosphate group of the cofactor, we used ^{31}P NMR spectroscopy (cf. Feldmann & Hull, 1977). This method allowed us to measure changes in the ionization of the 5′-phosphate group of pyridoxal-P bound to phosphorylase *b* or *a* upon transition from the inactive to the active conformation by adding either the allosteric activators AMP or AMP-S or by phosphorylating serine residue 14 with phosphorylase kinase and Mg^{2+}/ATP or Mg^{2+}/ATP-γ-S. In both cases, the transition from the inactive to the active enzyme was accompanied by deprotonation of the phosphate group of the cofactor, i.e., formation of the dianionic species within a protective environment of the active enzyme conformation. At first, a location of the active site at the subunit interface (Jones & Cowgill, 1971) seemed in line with X-ray diffraction data (Fletterick et al., 1976). When pyridoxal-P was bound at or near the active site, this location would have been compatible with the hydrophobic and protected character of the cofactor binding site apparent from the peculiar UV and fluorescence spectral properties of the pyridoxal-P Schiff base in phosphorylase *b* (Shaltiel & Cortijo, 1970; Cortijo & Shaltiel, 1970). Furthermore, it might explain why the monomer is inactive and dimer formation is a prerequisite for expression of activity (Feldmann et al., 1972). We decided, therefore, to assess by ^{31}P NMR the effect of dimer to monomer dissociation on the ionization of pyridoxal-P in phosphorylase hoping that this study would clarify the location of the cofactor

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[1] Abbreviations used: P, phosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; AMP-S, adenosine 5′-*O*-thiomonophosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); AMP, adenosine 5′-monophosphate; P_i, inorganic phosphate.

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and allow us to compare information obtained on the enzyme in solution with information on the enzyme in the crystalline state deduced from X-ray diffraction data. In the meantime, the data on the location of pyridoxal-P in phosphorylase were superceded by more recent X-ray diffraction data (Sygusch et al., 1977; Kasvinsky et al., 1978a,b; Weber et al., 1978; see also Feldmann et al., 1978) with which they are in full agreement. However, the data are still worth reporting because they bear on the ionization of the phosphate group of pyridoxal-P and its possible role for phosphorylase activity and provide, moreover, information on the molecular events underlying the conformational changes which accompany allosteric and covalent activation processes and their relation to the cofactor binding site. Finally experiments are reported with pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a* which show that the 5′-side chain of the cofactor also senses the presence of the inhibitory substrate analogue glucose.

Materials and Methods

Chemicals and Buffer. Nucleotides and sugar phosphates were products of Boehringer, Mannheim Corp. Pyridoxal-P, pyridoxal 5′-deoxymethylenephosphonate (a kind gift of Dr. O. Saiko), oyster glycogen, 1-amino-2-hydroxynaphthalene-4-sulfonic acid, cysteine monohydrochloride, and imidazole were purchased from Merck, Darmstadt. 3-(*N*-Morpholino)propanesulfonic acid, bovine serum albumin, and sodium dodecyl sulfate were obtained from Serva, Heidelberg, and succinic anhydride and EDTA from EGA-Chemie, Steinheim. ^{14}C-labeled succinic anhydride was purchased from New England Nuclear. Sephadex G-25 was obtained from Pharmacia and glycerol 2-phosphate from Roth, Karlsruhe.

Two buffer systems were used: buffer A contained 50 mM glycerol 2-phosphate and 50 mM 2-mercaptoethanol, pH 7.0; buffer B was 100 mM 3-(*N*-morpholino)propanesulfonic acid, 2 mM EDTA, and 50 mM 2-mercaptoethanol adjusted to the desired pH with NaOH or HCl.

Enzymes. Phosphorylase *b* was prepared according to the method of Fischer & Krebs (1958) from frozen rabbit skeletal muscle and three times recrystallized in 50 mM glycerol 2-phosphate buffer, pH 7.0, containing 30 mM cysteine by addition of 1 mM AMP and 10 mM Mg^{2+}. The enzyme was freed of AMP either by passage over activated charcoal or Sephadex G-25. Apophosphorylase *b* was prepared by the procedure of Shaltiel et al. (1966) and reconstituted with 0.5-fold excess of pyridoxal 5′-deoxymethylenephosphonate for 120 min at 25 °C (Vidgoff et al., 1974; Feldmann et al., 1976). A phosphorylase *a* derivative was prepared from AMP-free phosphorylase *b* (30 mg/mL) in buffer A containing 0.01 the volume of a solution of 0.1 M ATP-γ-S and of a 1 M magnesium acetate solution (Gratecos & Fischer, 1974). The pH was adjusted to 8.2 with 2 M Tris and the reaction started by addition of purified phosphorylase *b* kinase (0.25 mg/100 mg of phosphorylase *b*) (a kind gift of Dr. Jennissen, Bochum). After incubation for 45 min at 30 °C, the pH was brought to 7.0 with 1 N acetic acid. Phosphorylase *a* crystallized in the cold. For recrystallization, the centrifuged pellet was suspended in buffer A and dissolved by addition of 0.1 volume of 5 M NaCl. Following a further centrifugation at room temperature, crystallization was initiated in the supernatant after removal of NaCl by dialysis. Pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *b* was phosphorylated as described above but by using ATP instead of ATP-γ-S as phosphoryl donor.

Protein Determination. Protein concentration was determined spectrophotometrically by using an extinction coefficient $A_{280nm}^{1\%}$ of 13.2 (Kastenschmidt et al., 1968). Molar concentrations are expressed in terms of monomers of 97412 daltons (Titani et al., 1977). When protein concentration was measured by using the Lowry method (Lowry et al., 1951), bovine serum albumin was used as standard.

Activity Measurements. Phosphorylase activity was determined in the direction of glycogen synthesis (Illingworth & Cori, 1953). Measurements were performed in 100 mM maleate buffer, pH 6.5, containing 100 mM glucose 1-phosphate, 1% glycogen, 1 mM AMP, and 25 mM 2-mercaptoethanol. The reaction was started by addition of enzyme. Initial velocities were determined from the release of P_i in the first 2 min by the procedure of Fiske & Subbarow (1925) or by using an autoanalyzer (Haschke & Heilmeyer, 1972). Phosphorylase *b* had under those assay conditions specific activities of 80–85 μmol of P_i min^{-1} (mg of protein)$^{-1}$. After preincubation of phosphorylase *a* with glycogen for 15 min at 30 °C, it showed specific activities of 57–60 and 63–68 μmol of P_i min^{-1} (mg of protein)$^{-1}$ in the absence and presence of 1 mM AMP, respectively. In this case, the reaction was started by addition of glucose 1-phosphate. The specific activity of pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a* was 22 and 25 μmol of P_i min^{-1} mg^{-1} in the absence and presence of 1 mM AMP, respectively.

Succinylation (cf. Klotz, 1967). Freshly prepared, AMP-free phosphorylase *b* (7–10 mg/mL) in 50 mM glycerol 2-phosphate buffer (pH as desired) was treated with portions of 1.25–5 μL succinic anhydride (40 mg/mL) dissolved in peroxide-free dioxane under vigorous stirring at room temperature. For large-scale preparations, the calculated amount of succinic anhydride was successively added in 10-μL portions in 2-min intervals, and the pH of the enzyme solution was adjusted to 7.0 with 2 M Tris solution. The residual activity of succinylated phosphorylase was measured in 50 mM maleate buffer, pH 6.5, containing 20 mM mercaptoethanol and 1 mg/mL bovine serum albumin. For NMR and sedimentation velocity measurements as well as for the removal of excess radioactive succinic anhydride, the modified enzyme was precipitated with an equal volume of a saturated ammonium sulfate solution in the cold and the precipitate was collected by centrifugation and redissolved in buffer B as concentrated as possible. Excess ammonium sulfate and radioactive succinate were removed by chromatography on Sephadex G-25 (2.5 × 45 cm), preequilibrated with buffer B.

In order to determine the incorporation rate of radioactive succinic anhydride, AMP-free, native phosphorylase *b* was treated with [1,4-^{14}C]succinic anhydride (9.32 Ci/mol) and freed of excess succinate by gel chromatography. Radioactivity of enzyme aliquots was assayed in a Triton X-100 basal liquid scintillation cocktail and counted in a Packard Model 3380 Tri-Carb.

For the study of the resolution of pyridoxal-P from succinylated phosphorylase *b*, the enzyme was first succinylated under standard conditions yielding a residual enzymatic activity of up to 16%. The solution was divided into two equal portions. One portion was used directly for spectroscopic measurements in a Zeiss Model PMQII or a Cary Model 15 recording spectrophotometer. The second portion was resolved according to the method of Shaltiel et al. (1966) modified as follows. An equal volume of 0.4 M cysteine buffer, pH 6.2 (without imidazole), was incubated at 0 °C for 30 min with the enzyme solution. The modified apoenzyme was then precipitated with ammonium sulfate as described and excess salt was removed on a Sephadex G-25 column, preequilibrated with 50 mM glycerol 2-phosphate, pH 7.0. The effluent was

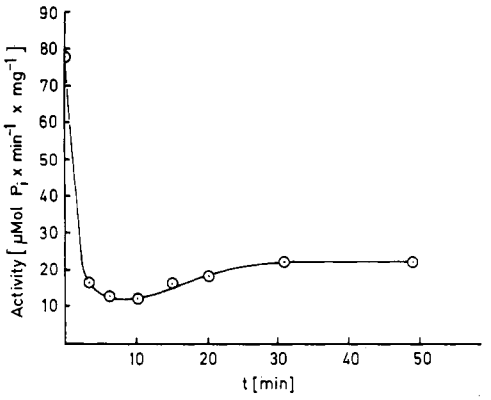

FIGURE 1: Inactivation of phosphorylase *b* by succinic anhydride. Conditions: phosphorylase *b*, 7 mg/mL; 5 μL of succinic anhydride (40 mg/mL) in anhydrous, peroxide-free dioxane; glycerol 2-phosphate buffer, pH 7.0, at room temperature.

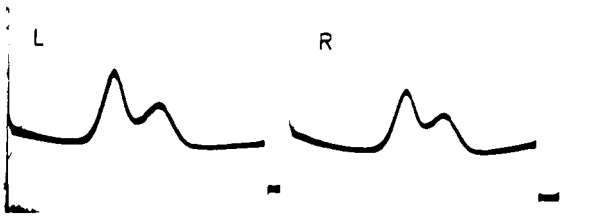

FIGURE 2: Sedimentation velocity pattern of partially succinylated phosphorylase *b* and *a*. Partially succinylated phosphorylase *b* and *a*, with a residual enzymatic activity of 16 and 6%, are shown in the left (L) and the right (R) panels, respectively. Conditions: protein concentration, 11 mg/mL in buffer B, pH 7.1 (L) and 7.3 (R); temperature, 20 °C; 60 000 rpm; Schlieren angle, 70°; single sector cell. Photographs were taken 60 min (L) and 48 min (R) after reaching maximal speed. The monomer–dimer distribution was estimated from the corresponding areas under the peaks corrected for radial dilution as described under Materials and Methods. Sedimentation was from right to left.

also analyzed spectrophotometrically and enzymatic activity determined after 1-h incubation with and without pyridoxal-P.

Ultracentrifugal Analyses. Sedimentation velocity experiments were performed at 20 °C in a Spinco Model E analytical ultracentrifuge equipped with a RTIC unit and a phase plate for the Schlieren optical system. Schlieren patterns were measured with a Leitz optical microcomparator. Single sector cells with 12-mm aluminium centerpieces and sapphire windows were employed. The enzyme concentration was 11 mg/mL in buffer B. The monomer–dimer distribution was determined from the corresponding area under the peaks (F_t) at the time t corrected for the radial dilution effect by multiplying the square of the distance of the peak (r_t^2) relative to the square of the distance of the meniscus (r_m^2) from the center of rotation since the concentration c is proportional to $F_t(r_t^2/r_m^2)$.

^{31}P NMR Measurements. Fourier transform ^{31}P NMR spectra were recorded at 72.86 MHz on a Bruker WH-180 wide-bore superconducting spectrometer. Enzyme samples (12 mL, 2–5 × 10^{-4} M per monomer) in 20-mm tubes were used. A concentric 5-mm NMR tube containing 99% D_2O was utilized for field/frequency lock. All spectra were recorded with broadband proton decoupling (0.4 W). In general, a 6000-Hz spectral width was acquired in 8192 data points with 60° pulse angle and 1.3-s repetition time. For enzyme samples, the exponential line broadening used prior to Fourier transformation was usually 10 Hz. Continuous air flow through the spectrometer probe head kept the temperature constant.

Results

Succinylation. Succinylation of phosphorylase *b* in 50 mM Tris buffer, pH 8.0, with solid succinic anhydride (Meighen & Schachman, 1970) led to rapid inactivation of the enzyme (Zeisel, 1973); however, the extent of incorporated label varied considerably. This disadvantage was overcome by preparing stock solutions of the reagent in anhydrous dioxane. Addition of up to 10% dioxane to phosphorylase *b* preparations did not alter the enzymatic activity within the accuracy of the assay. It should, however, be noted that the extent of succinylation could only be well controlled with enzyme preparations not older than 3–4 weeks. The overall time course of inactivation of phosphorylase *b* with succinic anhydride is shown in Figure 1. A rapid initial phase of inactivation ceasing after about 10 min is followed by a slow partial reactivation. Since the major part of added succinic anhydride is hydrolyzed, the possibility of a reactivation of the enzyme by succinate was investigated because anions are known to affect phosphorylase activity (cf. Appleman et al., 1966; Sealock & Graves, 1967; Stalmans & Hers, 1975). Phosphorylase *b* reacted for only 1 min with succinic anhydride, however, showed the same reactivation phase even though excess reagent and hydrolysis products were immediately removed by gel chromatography on Sephadex G-25. Thus, activation of the enzyme by succinate seems very unlikely. We therefore considered that reactivation could be due to rearrangement of the quaternary structure. In order to test this assumption, the sedimentation velocity pattern of partially succinylated phosphorylase *b* with a residual activity of 16% was determined in an analytical ultracentrifuge. The planimetric analysis of the monomer–dimer distribution (Figure 2L) indicated 40% monomer and 60% dimer for succinylated phosphorylase *b*. Even a 2-h preincubation with 1 mM AMP did not alter the monomer–dimer ratio, indicating that the succinylated monomer is frozen and/or might have lost its ability to bind AMP which normally induces reassociation.

In order to obtain comparable labeling patterns for the ^{31}P NMR experiments, succinylated phosphorylase *b* with 16% residual activity was converted into phosphorylase *a* by using [^{32}P]ATP-γ and phosphorylase *b* kinase. The degree of conversion was estimated by measuring the incorporation of ^{32}P. The same incorporation was found in the case of native and succinylated phosphorylase, indicating complete *b* to *a* conversion also for succinylated phosphorylase. Analysis of the sedimentation velocity pattern of succinylated phosphorylase *a* with 6% residual activity showed a monomer–dimer ratio of 43 to 57% (Figure 2R).

The incorporation of succinate was assessed by using radioactive succinic anhydride. For this purpose, phosphorylase *b* was incubated for 12 min with various amounts of ^{14}C-labeled reagent under standard conditions and excess hydrolyzed reagent removed by gel filtration. Preparations of succinylated phosphorylase *b* with 15% residual activity containing on the average 6 radioactively labeled lysyl residues out of a total of 48 lysyl residues per monomer (Titani et al., 1977) were used for ^{31}P NMR experiments as well as for ultracentrifugation studies. Succinylation depends on the concentration of uncharged ε-amino groups. Modification of phosphorylase *b* by succinic anhydride under standard conditions at different pHs from 5 to 7 showed increasing degree of succinylation with raising the pH as to be expected from the increased number of deprotonated ε-amino groups. Succinylation was carried out at pH 7 near the pH optimum of phosphorylase activity.

Since succinylated phosphorylase *b* dissociates partially (see Figure 2L), it seemed of interest to assess the perturbations of the tertiary structure upon succinylation by trying to remove pyridoxal-P from the modified enzyme with carbonyl reagents,

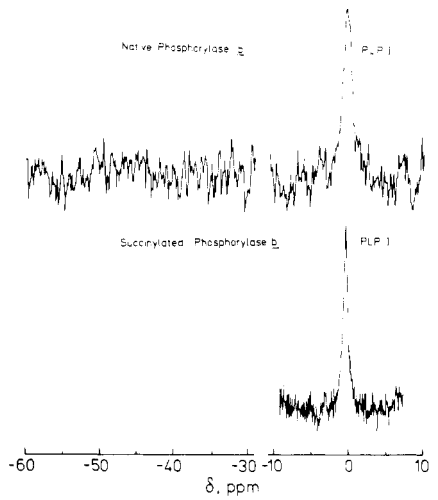

FIGURE 3: ^{31}P NMR spectra of native and succinylated phosphorylase *b*. Native phosphorylase *b*, 0.62 mM monomers, free of endogeneous AMP in buffer B, pH 7.1. The chemical shift of pyridoxal-P (form I) is −0.37 ppm relative to triethyl phosphate as external standard. The spectrum represents 4300 scans with repetition time 1.3 s and pulse width 30 μs. Succinylated, partially dissociated phosphorylase *b*, 0.29 mM monomers in buffer B, pH 7.23, with a residual activity of 15% relative to that of nonsuccinylated enzyme. The chemical shift of pyridoxal-P (form I) is −0.31 ppm. The spectrum represents 22 000 scans with repetition time 2 s, pulse width 30 μs, and 1200-Hz spectral width acquired in 4096 data points.

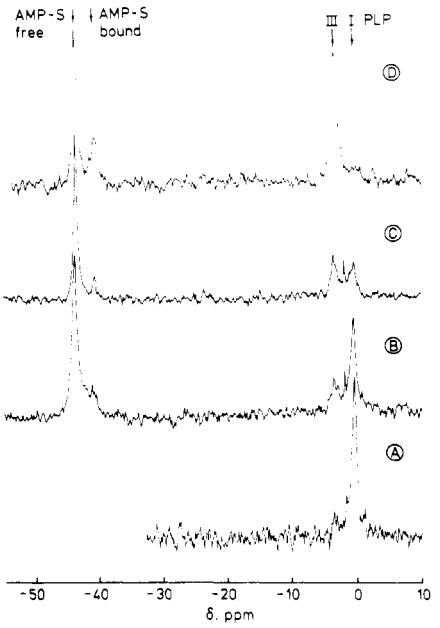

FIGURE 4: The effect of AMP-S and arsenate on the ^{31}P NMR spectra of native and succinylated phosphorylase *b*. Panels A, B, and C represent spectra of succinylated phosphorylase *b*, 0.2 mM monomers, in buffer B with a residual activity of 15%. These spectra were obtained with repetition time 1.3 s, pulse width 30 μs, and 6000-Hz spectral width acquired in 4096 data points. (A) Conditions: pH 7.12, 35 900 scans, in the absence of AMP-S and arsenate. The chemical shift of pyridoxal-P (form I) is −0.32 ppm. (B) Conditions: pH 7.05, in the presence of 0.7 mM AMP-S (3.5 mol/monomer). The chemical shifts are: pyridoxal-P, form I and III, −0.40 and −3.06 ppm, respectively; AMP-S, free, −43.40 ppm, and bound, −40.45 ppm, respectively. (C) Conditions: pH 7.29, same AMP-S concentration as in B but in the presence of 30 mM arsenate, 39 000 scans. The chemical shifts are: pyridoxal-P: form I, −0.5 ppm; form III, −3.62 ppm. AMP-S, free, −43.36 ppm, and bound, −40.41 ppm, respectively. (D) Native phosphorylase *b*, 0.5 mM monomers, in buffer B, pH 7.1, 1.5 mM AMP-S (3 AMP-S/monomer), 36 mM arsenate, 23 690 scans. The chemical shifts are: pyridoxal-P (form III), −3.47 ppm. AMP-S, free, −43.63 ppm, and bound, −40.42 ppm, respectively.

such as L-cysteine but in the absence of imidazole citrate as deforming reagent (Shaltiel et al., 1966). From the decrease of 330-nm absorption after treatment with L-cysteine, it was estimated that about 27% of the succinylated phosphorylase *b* was converted to apoenzyme as compared to 7% for native phosphorylase *b* (Pfeuffer et al., 1972). Assuming that the cofactor is only accessible to L-cysteine in the succinylated monomer, actually 67% of the latter would have been converted to apomonomer, although the UV spectra gave no indication that the environment of the cofactor binding site had become more polar. In order to learn whether succinylation influenced the ionization state of the phosphate group of pyridoxal-P, ^{31}P NMR measurements were carried out.

Ionization of the 5′-Phosphate of Pyridoxal-P. The ^{31}P NMR signals for P_i, glucose 1-phosphate, AMP, phosphoserine, and pyridoxal-P are all located in a narrow range of 4 ppm. The difficulty of overlapping signals could, however, be overcome by substituting AMP-S for AMP and arsenate for P_i. Furthermore, a phosphorylase *a* derivative containing thiophosphoseryl residues was prepared from phosphorylase *b* by using ATP-γ-S as substrate (Gratecos & Fischer, 1974; Feldmann & Hull, 1977). For the glucose experiments, a phosphorylase *a* derivative was used which contained pyridoxal 5′-deoxymethylenephosphonate but a phosphoseryl residue since both signals are well separated. A comparison of the ^{31}P NMR spectra of native and of succinylated, partially dissociated phosphorylase *b* is shown in Figure 3. The same chemical shift of the phosphate signal was seen in both cases, indicating that succinylation had no effect on the ionization of the phosphate group of pyridoxal-P in the inactive enzyme despite the ease with which part of the cofactor is resolved from the succinylated enzyme with L-cysteine. The signal at −0.37 ppm was previously assigned to the protonated form of the phosphate group of bound cofactor and designated as form I (Feldmann & Hull, 1977). It is pH independent in the absence of AMP and prior to phosphorylation of serine residue 14. At pH 7.2 where this experiment was carried out, more than 90% of the pyridoxal-P would have been expected to be in the dianionic form when contact with the solvent was possible. In this case, the signal should be displaced by about 4 ppm to lower fields. This also rules out that significant amounts of pyridoxal-P became detached from succinylated phosphorylase under the different conditions of the NMR experiments. Thus, it is concluded, in accordance with recent X-ray crystallographic evidence (Sygusch et al., 1977; Weber et al., 1978), that the active site is not at or near the subunit interface but is located in the inner core of the monomer. This conclusion is supported, although more indirectly, by the analysis of the effect of glucose on phosphorylase *a* reported below.

Activation of Succinylated Phosphorylase by AMP-S and Phosphorylation. Previous experiments had shown that the phosphate signal of the cofactor reports sensitively the transition from the inactive to the active conformation of phosphorylase *b* on binding of AMP-S or phosphorylation (Feldmann & Hull, 1977). The transition is facilitated by the simultaneous addition of the substrate analogue arsenate which enhances the affinity of the enzyme for AMP (Helmreich & Cori, 1964). Therefore, the effects of AMP and arsenate on partially succinylated phosphorylase *b* were studied and compared with the corresponding changes observed in native phosphorylase *b*. The spectrum in Figure 4A shows partially succinylated phosphorylase *b* in the absence of effector and substrate analogue at pH 7.12, indicating the protonated form I. The spectrum in Figure 4B was recorded at pH 7.05 with the succinylated enzyme in the presence of 0.7 mM AMP-S,

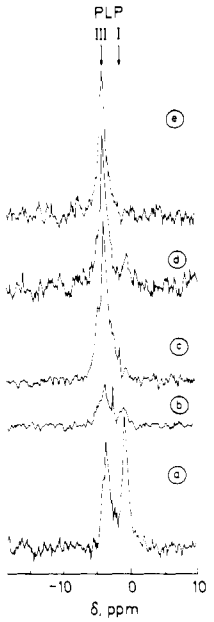

FIGURE 5: Changes in the ionization of pyridoxal-P of succinylated and native phosphorylase *a* containing thiophosphoseryl residues. Panels a and b represent spectra of succinylated phosphorylase. (a) Succinylated thiophosphoseryl phosphorylase *a*, 0.37 mM monomers, in buffer B, pH 7.35, with a residual activity of 6%. The spectrum represents 21 170 scans with repetition time 2.3 s, pulse width 25 μs, and 6000 Hz spectral width in 4096 data points. The chemical shifts for form I and III of pyridoxal-P are −0.44 and −3.2 ppm, respectively. (b) Succinylated thiophosphoseryl phosphorylase *a*, 0.39 mM monomers in buffer B, pH 7.35 containing 1.17 mM AMP-S (3 mol/monomer); 15 580 scans; instrument settings as in a. Chemical shifts: pyridoxal-P, form III, −3.35 ppm, and form I, −0.56 ppm. Panels c, d, and e represent spectra of nonsuccinylated phosphorylase *a*. (c) Thiophosphoserylphosphorylase *a*, 0.42 mM monomers, in buffer B, pH 6.5. The spectrum represents 23 950 scans with repetition time 3 s, pulse width 25 μs, and 6000 Hz spectral width in 8192 data points. The chemical shift for pyridoxal-P form III is −3.76 ppm. (d) Thiophosphoseryl phosphorylase *a*, 0.25 mM monomers, in buffer B containing 0.3 M NaCl, pH 7.67. The spectrum represents 28 300 scans with repetition time 1.3 s, and 7200 Hz spectra width in 2048 data points. The chemical shifts for forms I and III of pyridoxal-P are −0.30 and −3.75 ppm, respectively. (e) Addition of AMP-S (1.39 mol/monomers) to enzyme in panel d. The spectrum was taken at the same instrument settings given in d except 21 400 scans were collected. The chemical shift of form III of pyridoxal-P is −3.76 ppm.

corresponding to 3.5 mol/mol of monomer. The signal of AMP-S is exchange-broadened, indicating that free AMP-S is exchanging with enzyme-bound effector at a medium-fast rate. The binding of AMP-S induces a conformational change in the enzyme leading to a change of the cofactor signal. In addition to the still predominate form I of pyridoxal-P, form III with a downfield shift of −3.06 ppm was observed. Form III represents the fully deprotonated, dianionic form of the cofactor (cf. Feldmann & Hull, 1977). The spectrum of the same enzyme solution in the presence of AMP-S but with 30 mM arsenate is shown in Figure 4C. The enhanced affinity for the nucleotide effector caused by arsenate (Helmreich & Cori, 1964) and the resulting increased lifetime of the enzyme–effector complex are most likely responsible for the line narrowing of the AMP-S resonance. Concomitantly more pyridoxal-P has changed from form I to form III. The sharp signal between the resonance lines for forms I and III results most likely from a contamination of AMP-S with inorganic phosphate. The exchange between free and bound AMP-S is rapid enough to obtain an average T_1. Over 50% of the enzyme is present as enzyme–effector complex as can be calculated by integrating the AMP-S resonances. The concentrations of the enzyme–effector complex and of form III

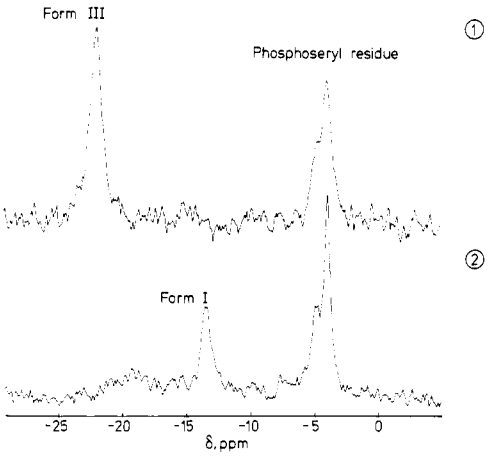

FIGURE 6: ^{31}P NMR spectra of pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a* in the absence and presence of glucose. (1) Pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a*, 0.4 mM monomers, in buffer B, pH 7.07, containing 200 mM NaCl. The spectrum represents 13 550 scans with repetition time 1.6 s, pulse width 25 μs, 4800 Hz spectral width in 8192 data points. The chemical shifts are: form III of pyridoxal 5′-deoxymethylenephosphonate, −21.6 ppm; phosphoseryl residue, free, −3.76 ppm, and bound, −4.58 ppm, respectively. (2) Pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a*, 0.29 mM monomers, in buffer B, pH 7.07, containing 200 mM NaCl and 100 mM glucose. The spectrum represents 38 901 scans with repetition time 1.4 s, pulse width 25 μs, and 3000 Hz spectral width in 8192 data points. The chemical shifts are: pyridoxal 5′-deoxymethylenephosphonate form I, −13.35 ppm; phosphoseryl residue, free, −3.76 ppm, and bound, −4.75 ppm, respectively.

correspond. Moreover, the distribution between form I and III correlate well with the monomer–dimer ratio (see Figure 2L). The spectrum of native phosphorylase *b* is shown in Figure 4D for comparison. A threefold excess of AMP-S per monomer in the presence of 36 mM arsenate suffices to obtain a fully active enzyme with only form III of the cofactor. The following experiments were carried out with native and succinylated phosphorylase *a* containing thiophosphoseryl residues. Spectra of succinylated and native phosphorylase *a* are presented in Figure 5a,b and 5c–e, respectively. For better comparison, only the phosphate signals of pyridoxal-P are shown. In native phosphorylase *a* at pH 6.5, only form III of pyridoxal-P was found (panel c). In contrast, the spectrum of succinylated phosphorylase *a* at pH 7.35 indicates that pyridoxal-P is in forms I and III but with form I predominating (panel a). When AMP-S (3 mol/monomer) was added to the succinylated enzyme, form III increased, but a complete conversion was not achieved with succinylated phosphorylase *a* (panel b). The sharp signal between the resonance lines for forms I and III is again attributed to contamination of AMP-S with inorganic phosphate. At pH 7.7, the main component in native phosphorylase *a* is form III but, in addition, 17% of pyridoxal-P is in the protonated, inactive conformation (form I) (panel d). In the presence of AMP-S, the small portion of form I disappeared in favor of form III (panel e).

Effect of Glucose. Glucose is a specific inhibitor of phosphorylase *a* (Cori et al., 1943). Addition of 50 mM glucose yielded a phosphorylase *a* binding AMP poorly ("T" form). Therefore, it was concluded that phosphorylase *a* is an allosteric enzyme like phosphorylase *b* with preferential binding of AMP and substrates to the "R" form and of the inhibitor glucose to the "T" form of the enzyme (Helmreich et al., 1967). Experiments with the pyridoxal 5′-deoxymethylenephosphonate derivative of phosphorylase *a* in the absence and presence of 100 mM glucose shown in Figure 6,1

and 6,2, respectively, corroborate the findings described above. In this phosphorylase *a* derivative, the pyridoxal-P analogue is likewise exclusively present in the "active" form III. Addition of the substrate analogue glucose caused, as to be expected, a partial conversion of the active form III of pyridoxal-P analogue to the inactive form I. Residual portions of form III are exchange-broadened since the line width of the fixed form III is $\Delta\nu_{1/2} \approx 55$ Hz. This exchange, however, does not occur with form I since it shows a normal and not an exchange-broadened signal. It rather occurs with form II as defined by Feldmann & Hull (1977). The question whether this behavior results from a "steric" rather than an "allosteric" interference[2] caused by glucose and the implication of this observation for a location of pyridoxal-P near the site to which glucose binds are addressed in the discussion. How AMP overcomes the glucose inhibition allosterically remains to be elucidated on a molecular basis.

Molecular Basis of Phosphorylase Activation by AMP and Phosphorylation. Activation of phosphorylase *b'* lacking the N-terminal phosphorylation site by AMP shows that the phosphorylation site is not required for AMP activation (Cori & Cori, 1945; Fischer et al., 1959). Subsequently Graves et al. (1968) showed desensitization of allosteric activation by AMP in phosphorylase *b'* due to the removal of the peptide containing Ser-14 which is phosphorylated in the *b* to *a* conversion. A study of the interaction of the phosphoseryl residue and the phosphate group of AMP with the enzyme might not only help toward an understanding of allosteric and covalent activation on a molecular basis, it might also offer an explanation for the vastly different requirements of native muscle phosphorylase *b* and *a* and of native and succinylated phosphorylase *a* for AMP to induce activity. An analysis of the thiophosphoseryl and AMP-S resonances was therefore carried out utilizing the experiments performed with succinylated and native phosphorylase *a*, shown in Figure 5. The spectra in panels α,β and γ,ϵ of Figure 7 were recorded under the same conditions as the corresponding spectra a,b and c,e in Figure 5. Experiment δ in Figure 7 was performed at different instrument settings but, otherwise, under the same conditions as that in Figure 5d. Near the pH optimum of the enzymatic reaction at pH 6.5, a single signal for the thiophosphoseryl residue was observed in native phosphorylase *a* (panel γ). The line width and the signal location indicate the fixation of the dianionic thiophosphate group in a salt bridge. When the pH is changed to 7.67, a second peak at lower field appears (panel δ), indicating that at this pH the interaction of the thiophosphoseryl residue with the amino acid side chains is partly abolished. Fixed and mobile thiophosphoseryl residues are in slow chemical exchange. It might be recalled that in this case a significant portion of pyridoxal-P is in the inactive enzyme form I (see Figure 5d) which can be converted to the active enzyme form III with concomitant disappearance of the resonance line for the mobile thiophosphoseryl residue upon adding AMP-S (compare 5e and 7ϵ). The resonance for AMP-S is split into two signals at pH 7.67, one for bound and the other for free AMP-S. The thiophosphoseryl residue interacting with the enzyme is most probably fixed in a salt bridge. In addition, AMP-S at a molar ratio of 1 mol/monomer is bound as dianion most likely also in a salt bridge. Suitable partners in forming such salt bridges could be arginine residues.[2] The role of arginines for phosphorylase activity is

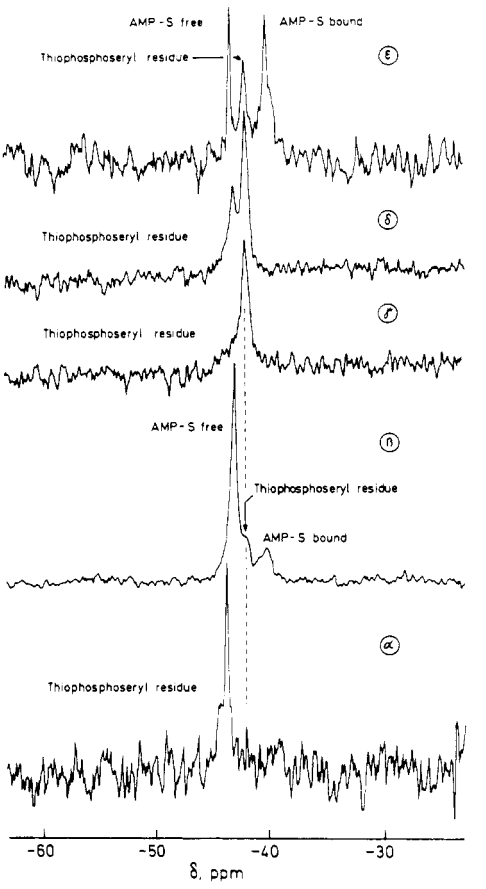

FIGURE 7: Effects of AMP-S and pH on the ^{31}P NMR spectra of the thiophosphoseryl residue of succinylated and native phosphorylase a. The spectra in panels α and β for succinylated γ and ϵ for native phosphorylase *a* were taken with the same enzyme preparations and conditions given in Figures 5 a,b and c and e, respectively. The spectrum in panel δ was again recorded with the same enzyme solution used in Figure 5d but at different instrument settings: 22 803 scans with repetition time 3.1 s, pulse width 25 μs and 6000 Hz spectral width in 8192 data points. The chemical shifts are: thiophosphoseryl residue bound in α, −44.2 ppm, and −42.24 ppm in γ. AMP-S (panel ϵ): free, −43.6 ppm; bound, −40.5 ppm. AMP-S (panel β): free, −43.46 ppm; bound, −40.5 ppm. Thiophosphoseryl residue (panel β): −42.2 ppm as shoulder.

supported by the inactivation experiments with butanedione (Li et al., 1977). Since the thiophosphoseryl residue and the thiophosphate group of the nucleotide are both attached simultaneously to the enzyme in stoichiometric amounts, they must be triggering the active enzyme conformation from two different amino acid side chains (arginines) (see also Madsen et al., 1978). The nucleotide binding site would be the strong activator binding site adjacent to serine-14 (Kasvinsky et al., 1978a). Moreover, a comparison of the spectra in Figures 7δ and 7ϵ suggests that the attack of each of the phosphates at a different domain of the enzyme is cooperative in the sense that both phosphates mutually assist each other in forcing the enzyme in the active conformation. Morange et al. (1976) have pointed to the importance of the 5'-phosphate moiety in determining binding specificity of AMP to muscle phosphorylase and have distinguished two classes of nucleotide binding sites (weak and strong sites). When the pH is raised from 6.5 to 7.67, the dianionic form of the thiophosphoseryl residue becomes partly detached and more mobile. This may result from a more mobile enzyme structure making attachment more difficult or from deprotonation of positively charged amino acid side chains in the domains to which the thiophosphoseryl residues bind. On adding AMP-S at the same pH, the thiophosphoseryl residue can now again be fixed

[2] As this paper was in review, an interesting report appeared by Madsen et al. (1978), who demonstrated by X-ray crystallography a large conformational change on addition of glucose to muscle phosphorylase *a* which inter alia exposed the Ser-14 phosphate bonded to Arg-69 on helix 49–75.

completely despite the unfavorable, alkaline pH.

Examining the location and the line width of the thiophosphoseryl residue in succinylated phosphorylase *a* (see Figure 7α), it can be concluded that in the modified enzyme most of the thiophosphoseryl residues are unbound and mobile. When any interaction with the enzyme should occur, then only a minor fraction is involved for a short time. In this context, it is noteworthy that the activity of succinylated phosphorylase *a* rises from less than 1 to 18 units upon addition of AMP compared with an increase of only 10% for the native enzyme. Reasons for this difference in AMP dependence for activation inferred from Figure 7β are discussed below.

Discussion

Form I was previously assigned to the protonated phosphate of pyridoxal-P in the inactive conformation of phosphorylase and form III to the fully deprotonated (dianionic) phosphate in the active conformation. For native dimeric phosphorylase *b*, a threefold excess of AMP-S over binding sites sufficed to force the enzyme in the presence of 30 mM arsenate completely into form III thought to represent the active enzyme (Feldmann & Hull, 1977; Figure 4D of this paper). Interestingly, in potato phosphorylase, whose activity is neither allosterically nor covalently regulated, the phosphate group of the cofactor is present in the dianionic form III in an enzyme free of primer and without ligands (H. W. Klein and E. J. M. Helmreich, unpublished results). This is in contrast to succinylated phosphorylase *b*. Although AMP-S is bound more weakly to succinylated phosphorylase, even in the presence of arsenate, we estimate that still about 55% of the enzyme–effector complex was formed at a 3.5-fold excess of allosteric nucleotide. But despite binding of the nucleotide, the transition of the inactive to the active enzyme was incomplete; although binding of AMP-S to succinylated phosphorylase resulted in a decline of form I and the appearance of form III, the latter was only about one-half of that present in native phosphorylase under comparable conditions. The same applies to succinylated phosphorylase *a*. Active, native phosphorylase *a* contains at neutral pH nearly exclusively form III, whereas in the case of succinylated phosphorylase *a* the conversion of the inactive form I into the active form III on phosphorylation was likewise incomplete (see Figure 5). The final distribution of form I and III of the phosphate of pyridoxal-P in succinylated phosphorylase *b* and *a* on allosteric or covalent activation roughly corresponds to the monomer–dimer distribution in these preparations. But assuming that the monomer is inactive, a postulate which is well supported (Feldmann et al., 1972, 1976), and taking into account that 40% of succinylated phosphorylases were monomeric under the conditions of these experiments, one would have expected an activity loss of 40%, whereas actually 85% activity were lost. Thus, monomer formation alone cannot account for the loss of activity. Quite likely, a lysyl residue essential for activity is blocked in succinylated phosphorylase. To clarify this point, radioactively labeled succinylated peptides were isolated for sequence analysis. The results will be reported later.

Another point to be discussed is the much greater dependence of succinylated phosphorylase *a* on AMP for activity as compared with the native enzyme. The change in AMP requirement might be due to a perturbation of the enzyme structure which could be responsible for the impaired binding of the thiophosphoseryl residue in succinylated phosphorylase *a*. The thiophosphate must apparently form a stable salt bridge at the regulatory domain of the enzyme to stabilize the active conformation of phosphorylase *a*. Incidentally, Sealock & Graves (1967) had already suggested that differences in the properties of phosphorylases *b* and *a* might be due to an interaction of the covalently bound phosphate of phosphorylase *a* with a specific protein site near the surface of the molecule which is particularly sensitive to the ionic character of its environment. The experiments with native phosphorylase *a* in Figures 7γ and 7ϵ at different pHs show, in accordance with the X-ray crystallographic evidence (see Figure 1 of Fletterick et al., 1976), that the binding of the thiophosphate of AMP-S and the thiophosphoseryl is not mutually exclusive. Hence, both phosphates bind to different but cooperative sites. The location and the small line width of the thiophosphoseryl resonance in succinylated phosphorylase *a* (Figure 7α) indicated that the residue is more mobile than in native phosphorylase *a*. When AMP-S was bound to the succinylated enzyme, then the thiophosphoseryl residue became again attached more strongly (Figure 7β). While this provides a reasonable explanation for the greater dependence of succinylated phosphorylase *a* on AMP for expression of activity, it is a matter of speculation how succinylation could interfere with the interaction of the thiophosphoseryl residue and the enzyme. The effect of AMP-S makes it unlikely that succinylation modifies directly a critical lysyl residue to which the phosphoseryl is attached in phosphorylase *a*. On the other hand, the possibility that succinylation perturbs the structure of the binding domain by making a positively charged amino group (arginine) less accessible for the thiophosphoseryl residue received some support from the data in Figure 7β. The resonance line of the thiophosphoseryl residue in succinylated phosphorylase *a* is shifted on binding of AMP-S from −44.2 ppm (Figure 7α) to −42.2 ppm (Figure 7β) and is now located as a shoulder on the right side of the resonance line for free AMP-S. This indicates that the thiophosphoseryl residue can be bound to succinylated phosphorylase *a* on interaction with AMP-S. Thus, AMP-S partly can rearrange the disarrayed structure of the succinylated enzyme and enables reattachment of the thiophosphate, although AMP-S is bound more weakly to succinylated phosphorylase *a* and its residence time is shorter than with native phosphorylase *a*.

One of the more surprising findings of the earlier work by Helmreich et al. (1967) was that glucose 1-P was the only substrate which could overcome the glucose inhibition of phosphorylase *a*. Interestingly, inhibition by glucose is also specific, the α form being more inhibitory than the β form (Cori & Cori, 1940). Hence, we concluded that the glucose moiety either free or combined with inorganic phosphate undergoes specific binding at the active α-glucose 1-P site (cf. Cori et al., 1943; Helmreich et al., 1967). This conclusion is in accordance with the ^{31}P NMR data in Figure 6 which show the appearance of a phosphate resonance of the cofactor analogue in phosphorylase *a* on addition of glucose during the conversion of the active form III to the inactive form I. This broad signal appears to arise specifically on binding of glucose, indicating a change in the ionization of the phosphate of the cofactor in response to the binding of the substrate analogue to the active site. This agrees with the structural allocation of the glucose binding site by X-ray diffraction (Kasvinsky et al., 1978a). Further studies of this glucose effect seem worthwhile, because they may give insight into interactions of the phosphate of the cofactor with glucosyl residues during catalysis. The narrower line width of the phosphoseryl resonance in the glucose-inhibited enzyme (see Figure 6,2) could reflect the structural change recently seen by Madsen et al. (1978) in the 2.5-Å electron density map.[2]

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