Solid state ^{31}P cross-polarization/magic angle sample spinning nuclear magnetic resonance studies of crystalline glycogen phosphorylase b

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ABSTRACT ^{31}P cross-polarization/magic angle sample spinning nuclear magnetic resonance spectra have been obtained for pyridoxal 5′-phosphate (PLP) bound to glycogen phosphorylase b (GPb) in two different crystalline forms, monoclinic and tetragonal. Analysis of the intensities of the spinning sidebands in the nuclear magnetic resonance spectra has enabled estimates of the principal values of the ^{31}P chemical shift tensors to be obtained. Differences between the two sets of values suggest differences in the environment of the phosphate moiety of the pyridoxal phosphate in the two crystalline forms. The tensor for the tetragonal crystalline form, T state GPb, is fully consistent with those found for dianionic phosphate groups in model compounds. The spectrum for the monoclinic crystalline form, R state GPb, although closer to that of dianionic than monoanionic model phosphate compounds, deviates significantly from that expected for a simple dianion or monoanion. This is likely to result from specific interactions between the PLP phosphate group and residues in its binding site in the protein. A possible explanation for the spectrum of the monoclinic crystals is that the shift tensor is averaged by a proton exchange process between different ionization states of the PLP associated with the presence of a sulfate ion bound in the vicinity of the PLP.

INTRODUCTION

Glycogen phosphorylase is an allosteric protein that catalyzes the first step in the degradation of glycogen, involving the phosphorylysis of glycogen to produce glucose-1-P (1). The protein consists of identical subunits of molecular weights of ~100 kD assembled in dimers or tetramers depending on the conditions under which the protein exists. For example, in the presence of activators, glycogen phosphorylase dimers associate to form tetramers, but these dissociate back to dimers in the presence of glycogen or oligosaccharide. Evidence for dimer and tetramer formation has been reviewed by Dombrádi (2).

Glycogen phosphorylase may exist in an active conformation (R state) or an inactive conformation (T state). Active conformations can be achieved by two separate methods that involve different modifications to glycogen phosphorylase b (GPb).[1] The first method is a covalent modification of GPb to form glycogen phosphorylase a. The modification is a phosphorylation of the serine-14 residue of GPb catalyzed by phosphorylase kinase. The second method of activation is by a non-covalent association of GPb with an allosteric effector. Studies on the allosteric activation by effectors have been reviewed recently (3).

Several allosteric effector sites have been characterized, and varying extents of activation can be achieved with different effector molecules. For example, phosphorylase b can be activated by the non-covalent association of the enzyme with the allosteric effector adenosine 5′-monophosphate (AMP) to an active conformation with high substrate affinity (4). However, in the presence of the effector inosine 5′-monophosphate (IMP), a weak activation of phosphorylase b occurs with no increase in affinity of the enzyme for substrate. Inactive forms of the protein have been found to result on addition of one of a number of inhibitors; these include caffeine, adenosine diphosphate, adenosine triphosphate, glucose-6-P, and glucose (5–9).

Crystal structures for the protein from rabbit skeletal muscle in several different conformations have been determined. Crystals were first obtained in a tetragonal space group $P4_32_12$ in the presence of IMP, and a crystal structure was refined to 6 Å (10). Further refinements of this crystal form to 3 (11), 2.25 (12), and 1.9 Å (13) have followed. Crystallization from a 1.1 M ammonium sulfate solution produces a monoclinic form, space group $P2_1$, the structure of which has been solved and refined to 2.9 Å (14). As yet, no structure of GPb with AMP has been obtained, a result attributed to the decrease in solubility and tendency to aggregation when the protein is activated (3). However, a structure of phosphorylase b complexed with a modified cofactor, pyridoxal pyrophosphate, and with AMP has been reported (15). In addition, two different structures of glycogen phosphorylase a have been solved: one in the presence of the inhibitor glucose has been refined to 2.5 Å (16) and one in the presence of ammonium sulfate to 2.9 Å resolution (17).

In the tetragonal form the GPb is a dimer, whereas the monoclinic form is a tetramer. However, the overall to-

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[1] *Abbreviations used in this paper:* AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-*O*-thiomonophosphate; BES, (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid); CP, cross-polarization; CSA, chemical shift anisotropy; GPb, glycogen phosphorylase b; IMP, inosine 5′-monophosphate; MAS, magic angle sample spinning; NMR, nuclear magnetic resonance; PLP, pyridoxal phosphate.

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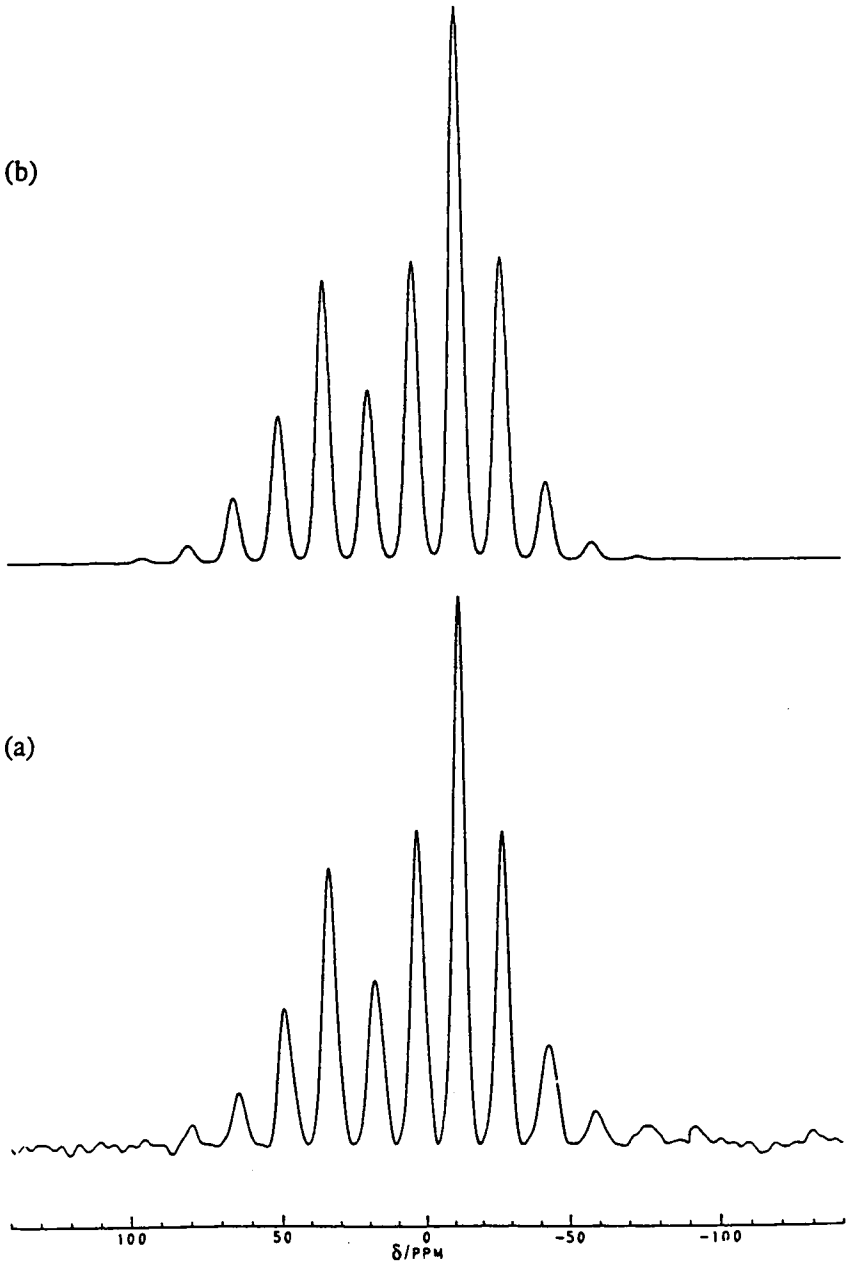

FIGURE 1 (*a*) ^{31}P CP/MAS NMR spectrum of the tetragonal crystalline form of GPb at 161.98 MHz. 40,000 transients were collected using a 200 mg protein sample. The spinning speed was 2.48 kHz. (*b*) Simulation of the spectrum from tensor components of $\delta_{11} = 66$ ppm, $\delta_{22} = -15$ ppm, $\delta_{33} = -41$ ppm, and $\delta_{iso} = 3.4$ ppm; and Lorentzian broadening of 50 Hz and Gaussian broadening of 800 Hz was used.

pology of the subunits in the two crystalline forms is similar. Each subunit consists of two domains: one consisting of residues 19–484 and the other of residues 485–842. The significant differences between the two structures are in those regions that are important for allosteric response or are in regions that are part of the tetramer interface of the monoclinic form. The N-terminal 18 residues are ordered in the monoclinic crystals, an active state of the protein, but disordered in the tetragonal crystals, an effectively inactive state of the protein. Small structural changes are observed at the ligand-binding sites and the subunit interface regions in the monoclinic form in comparison with the tetragonal form; however, no relative movements between the two domains of the subunit were found. Significant differences at the allosteric effector sites and the phosphorylated serine residue site (Ser14) are thought to be a consequence of extensive quaternary structural changes. In addition, some changes at the catalytic site and inhibitor site can be seen and are attributed to indirect effects of conformational changes (14).

Activity studies on phosphorylase reconstituted with modified pyridoxal phosphate (PLP) identified the PLP as an essential cofactor for activation (18). PLP is covalently bound to the protein, the aldehydic group of the cofactor forming a Schiff base linkage to Lys680. The

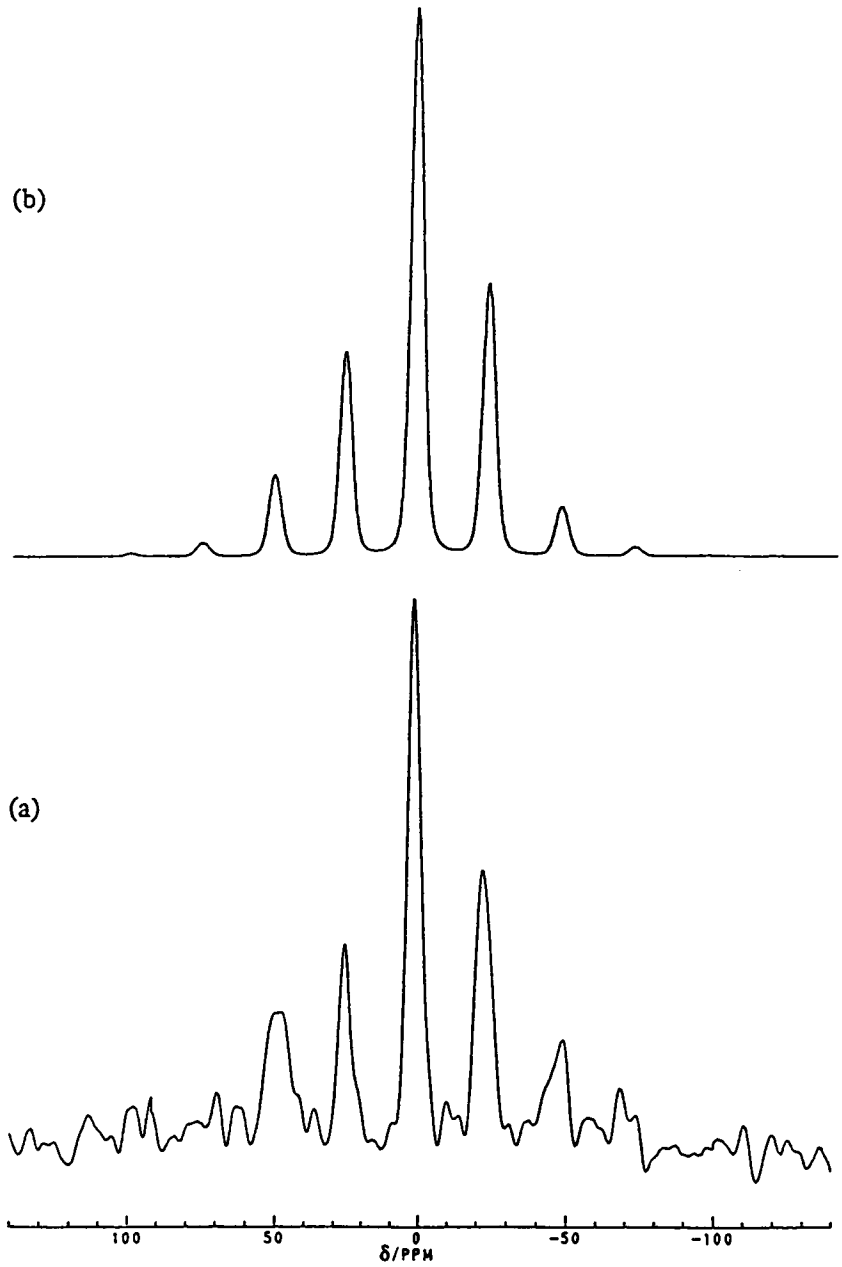

FIGURE 2 (*a*) ^{31}P CP/MAS NMR spectrum of the monoclinic crystalline form of GPb at 161.98 MHz. 100,000 transients were collected using a 50 mg protein sample. The spinning speed was 3.95 kHz. (*b*) Simulation of the spectrum from tensor components of δ_{11} = 54 ppm, δ_{22} = −5 ppm, δ_{33} = −42 ppm, and δ_{iso} = 2.0 ppm; and Lorentzian broadening of 200 Hz and Gaussian broadening of 700 Hz was used.

5′-phosphate was found to be of particular importance, and ^{31}P nuclear magnetic resonance (NMR) studies have shown that the resonance of this group shifts 3 ppm downfield on activation of the protein (19–21). These studies of the protein in solution have been interpreted to show that the PLP phosphate group is monoanionic when the protein is inactive and that the transformation to an active protein is accompanied by a deprotonation of the phosphate group to a dianionic state.

In this study we describe ^{31}P magic angle sample spinning (MAS) NMR experiments on both the monoclinic and tetragonal crystal forms of phosphorylase. These forms were chosen to be identical to those whose structures had been solved and are thought to represent active (R) and inactive (T) states of the protein and to provide an opportunity to correlate the observations of solution behavior with the crystallographic information.

MATERIALS AND METHODS

The nucleotides used in the preparation and purification of the protein and for ^{31}P NMR studies were purchased from Sigma Chemical Co. Ltd. (Poole, UK). For ^{31}P NMR studies the nucleotides were used either as received or were recrystallized from water or water/methanol

TABLE 1 **Isotropic and anisotropic ^{31}P NMR chemical shift data**

	^{31}P solid state NMR isotropic chemical shift	δ_{11}	δ_{22}	δ_{33}	Δ	η
	ppm					
Tetragonal crystalline form of GPb	3.4 ± 0.2	66 ± 1	−15 ± 3	−41 ± 3	107 ± 4	0.42 ± 0.03
Monoclinic crystalline form of GPb	2.0 ± 0.2	54 ± 2	−5 ± 8	−42 ± 8	96 ± 10	0.7 ± 0.1
PLP (free acid)	−1.72 ± 0.02	67 ± 3	7 ± 3	−79 ± 1	146 ± 4	0.78 ± 0.02
IMP (disodium salt)	2.71 ± 0.05	64 ± 1	−16 ± 3	−40 ± 3	104 ± 4	0.39 ± 0.03

mixtures. The nucleotides were confirmed to be of the same crystalline form as in the literature structures by x-ray powder diffraction.

Purification of GPb and crystal growth

GPb was isolated from rabbit skeletal muscle as first reported by Fischer and Krebs (22) with modifications described by Barford (23). Two methods were used to obtain crystalline protein. Tetragonal crystals were grown in the presence of 2 mM IMP (10). Crystals were obtained after 3 wk and rinsed extensively in a phosphate free buffer to remove free and non-covalently bound phosphates (at 2 mM, IMP is bound with very low occupancy [24]). This was achieved by soaking the crystals in a buffer of 10 mM (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) (BES), 0.5 mM ethylenediaminetetraacetate, 0.02% NaN_3, at pH 6.8 for 3 h and then removing the buffer and replacing it with a fresh buffer. The rinsing was performed six times over 2 d.

Monoclinic crystals were grown in a 1.1 M ammonium sulfate solution (14). Crystals were obtained after 1 month and were again rinsed extensively in a phosphate free buffer to remove free and non-covalently bound phosphates. This was achieved by dialyzing in a buffer of 10 mM BES, 1 M ammonium sulfate, pH 7.5, at 16°C for 2 d, with fresh buffer each day. The crystals were then rinsed by the repeated removal and addition of phosphate free buffer every hour for 5 h. The crystals were then left in buffer overnight.

The crystals were verified to be of the correct crystalline form by checking their morphology under a microscope. The protein was packed into a zirconia rotor after removing the surface solvent on filter paper.

Solid state NMR spectroscopy

^{31}P solid state NMR spectra of GPb and some of the model phosphate compounds were recorded on a (model MSL400; Bruker Instruments, Karlsruhe, Germany) pulse spectrometer with a 9.4 T wide bore superconducting solenoid magnet (Oxford Instruments, Oxford, UK) operating at a frequency of 400.13 MHz for ^{1}H. The instrument was equipped with an Aspect 3000 data system. The ^{31}P NMR spectra were recorded at a frequency of 161.98 MHz using a multinuclear, proton-enhanced, double-bearing MAS probe and a high power proton decoupler. All the spectra were referenced externally to 85% H_3PO_4 at 0 ppm, positive chemical shift values being taken as downfield of this reference.

Solid state NMR techniques of cross polarization (CP) along with MAS and high power proton decoupling were used to obtain all the spectra. The samples were packed into 7 mm diameter zirconia rotors with Kel-F tops. Typical rf fields used for ^{1}H were 0.9 mT (ω_1 = 38.5 kHz), resulting in a 90° pulse length of 6.5 μs. Contact times of 1 ms were used with a recycle delay of 3 s. Typical MAS speeds were 2–4 kHz. The samples were spun with great care to ensure stability in the spinning rate to within ±3 Hz. All spectra were recorded at ambient temperature.

Additionally, ^{31}P CP/MAS NMR spectra of the nucleotides were also recorded on a Bruker MSL200 pulse spectrometer with a 4.7 T wide bore magnet operating at a frequency of 200.13 MHz for ^{1}H, 81.02 MHz for ^{31}P, using a similar probe. Typical rf fields used for ^{1}H on the MSL200 were 1.3 mT (ω_1 = 55.5 kHz), resulting in a 90° pulse length of 4.5 μs. Approximately 100–500 transients were taken for the spectra of nucleotides and ~200 mg of sample used in the experiments.

Determination of chemical shift tensors

The spinning sideband manifold of the MAS NMR spectrum contains information regarding the chemical shift tensor. Initial values for the principal components of the chemical shift anisotropy (CSA) tensor, δ_{11}, δ_{22}, δ_{33}, were determined using a computer program written by Dr. M. M. Maricq (25) and modified by Dr. N. J. Clayden (25*a*). (The convention $\delta_{11} > \delta_{22} > \delta_{33}$ is used.) The program is based on the moment analysis of Maricq and Waugh (25) and uses the intensity profile of the MAS NMR spectrum and the isotropic chemical shift value, δ_{iso}. The values of the principal components were then put through an iterative test using a downhill simplex method of analysis to minimize the difference between the intensity profile of the experimental and simulated spectra using a program FSPIN written by Dr. N. J. Clayden and modified by Dr. J. M. Twyman (Oxford University). The tensor information was then used to evaluate the MAS NMR spectrum at any particular MAS rate. Slight modifications to the tensor were made to simulate a spectrum that best represented the experimental spectrum. The chemical shift anisotropy term, Δ, which is the spread of the CSA powder pattern, is defined in terms of the principal components as

$$\Delta = \delta_{11} - \delta_{33}.$$

The asymmetry parameter is defined by

$$\eta = (\delta_{11} - \delta_{22})/(\delta_{iso} - \delta_{33}) \quad \text{if} \quad (\delta_{iso} - \delta_{33}) > (\delta_{11} - \delta_{iso}),$$

and by

$$\eta = (\delta_{22} - \delta_{33})/(\delta_{11} - \delta_{iso}) \quad \text{if} \quad (\delta_{iso} - \delta_{33}) < (\delta_{11} - \delta_{iso}).$$

RESULTS

^{31}P CP/MAS NMR spectra of the tetragonal and monoclinic crystalline forms of GPb are shown in Figs. 1 *a* and 2 *a*, respectively. Despite the broad linewidths and the limited signal-to-noise ratios associated with detecting one ^{31}P nucleus in a protein of 100 kD molecular weight,

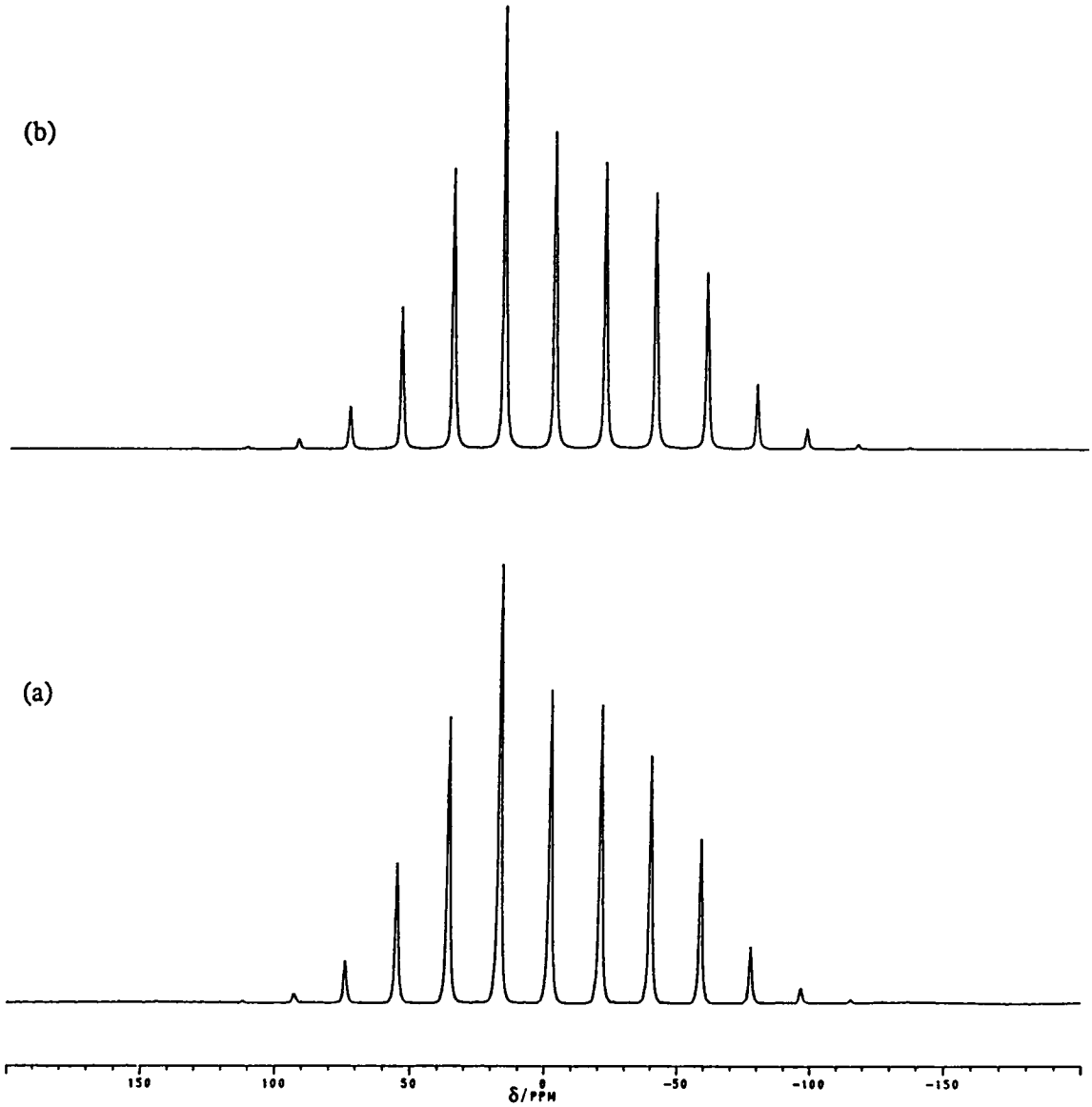

FIGURE 3 (*a*) ^{31}P CP/MAS NMR spectrum of the free acid of pyridoxal 5'-phosphate (PLP) at 81.02 MHz. 60 transients were collected using a 200 mg sample. The spinning speed was 1.53 kHz. (*b*) Simulation of the spectrum from tensor components of δ_{11} = 67 ppm, δ_{22} = 7 ppm, δ_{33} = −79 ppm, and δ_{iso} = −1.72 ppm; and Lorentzian broadening of 50 Hz and Gaussian broadening of 70 Hz was used.

the spectra were of sufficient quality to show clearly up to fourth and second order sidebands, respectively, for the two crystal forms. The intensities of the sidebands were sufficiently well defined to be used in analyses to determine principal values of the chemical shift tensors; the results are shown in Table 1. The resulting tensor values were used to simulate MAS spectra for the respective crystalline forms of the protein. These spectra are shown in Figs. 1 *b* and 2 *b* for the tetragonal and monoclinic crystalline forms, respectively.

The spectra are typical of MAS NMR with a single isotropic peak in each spectrum flanked on either side by a series of sidebands at intervals of the spinning frequency. The two spectra were acquired at different spinning speeds, 2.48 and 3.95 kHz, for the tetragonal and monoclinic forms, respectively. The conditions under which the monoclinic crystals are grown are more sensitive than those for the tetragonal form, resulting in difficulties in producing large quantities of material for study. Only 50 mg of the monoclinic form was used, whereas 200 mg of the tetragonal form was used in the NMR experiments. A higher MAS rate was used for the monoclinic crystals to optimize the signal-to-noise ratio, albeit at the expense of fewer sidebands for analysis. Repeated spectroscopy on the same batch of material showed increased resonance linewidths, indicating that the crystallinity of the protein crystals degrades within ~1 wk of removal of crystals from the mother liquor. The phosphate of the PLP in both tetragonal and monoclinic crystalline forms is buried ~15 Å from the surface of the enzyme and is rather shielded from the bulk water of the solvent (13, 14); removal of the protein crystals

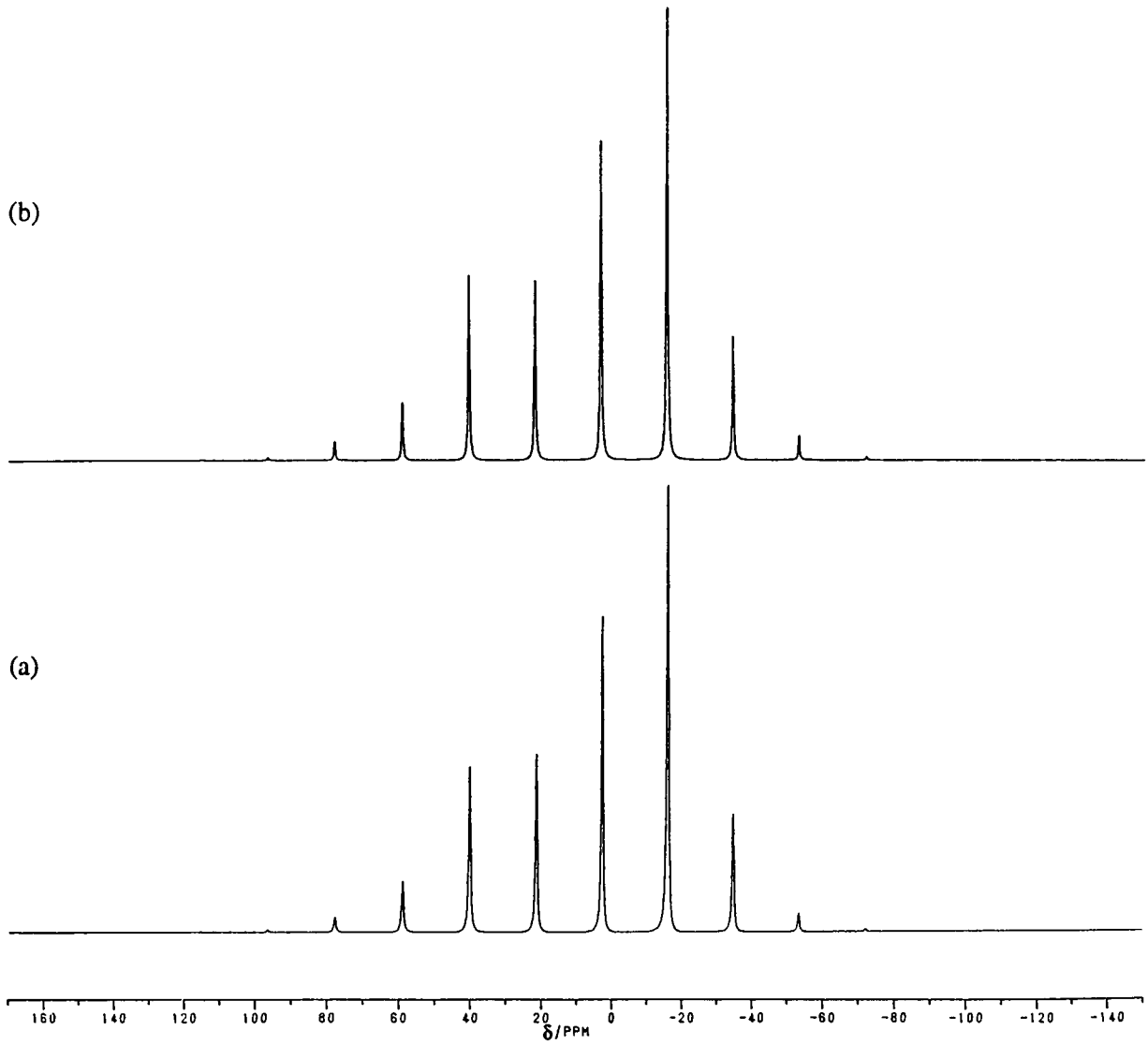

FIGURE 4 (*a*) ^{31}P CP/MAS NMR spectrum of the disodium salt of inosine 5′-monophosphate (IMP) at 161.98 MHz. 100 transients were collected using a 200 mg sample. The spinning speed was 3.03 kHz. (*b*) Simulation of the spectrum from tensor components of δ_{11} = 64 ppm, δ_{22} = −16 ppm, δ_{33} = −40 ppm, and δ_{iso} = 2.71 ppm; and Lorentzian broadening of 70 Hz and Gaussian broadening of 30 Hz was used.

from the solvent is therefore unlikely to remove essential water molecules needed to maintain the conformation about the PLP.

The rather broad inhomogeneous linewidths of ~800 Hz and the Gaussian lineshape of the resonances of the fresh crystals are presumed to result from a spread of environments for the phosphate group in the crystalline protein. The isotropic chemical shifts could, however, be determined to within ~0.2 ppm; they were 3.4 and 2.0 ppm for the tetragonal and monoclinic forms, respectively.

Several mononucleotides were studied as model phosphate monoester compounds for tensor analyses. The nucleotides selected for experiments all had known crystal structures. Some tensor analyses have already appeared in the literature for mononucleotides (26), but we have repeated a number of these and studied some new ones to test our method of tensor determination. Disodium salts of the nucleotides were used for model dianionic phosphate monoesters. Free acids were used for model monoanionic phosphate monoesters as structure determination has shown most to be zwitterionic, such that the phosphate group is a monoanion and the base is protonated (27–34).

The crystalline nucleotides have much narrower NMR linewidths of typically <100 Hz in comparison with 800 Hz for the protein spectra, suggesting a greater homogeneity of the environment of the phosphate group in the small molecule crystals than in the protein. The ^{31}P CP/MAS NMR spectrum of the free acid of pyridoxal 5′-monophosphate, in its monoanionic state (31), is shown in Fig. 3 *a*, and the simulated spectrum, from the tensors determined and listed in Table 1, is shown in Fig. 3 *b*. No crystal structure is available for the dianionic

form of this compound. The principal values of the tensor for the disodium salt of IMP, an example of one of the model dianionic phosphate monoester compounds, were however determined and are listed in Table 1. The experimental and simulated ^{31}P CP/MAS NMR spectra are shown in Fig. 4, *a* and *b*, respectively.

DISCUSSION

^{31}P NMR chemical shifts of organic phosphates in solution have been empirically correlated to O-P-O bond angles, bond orders, and substituent electronegativities (35, 36). Protonation or esterification of the tetrahedral PO_4^{3-} group perturbs the electron density distribution about the phosphorus nucleus and alters the O-P-O bond angles and P-O bond lengths. The chemical shift is therefore dependent on the overall ionization state of the phosphate group.

Examination of isotropic chemical shifts in solution and in the solid state of monoanionic and dianionic phosphate monoesters presented here and in the literature (37–45) reveals a general shift downfield of ~5 ppm on changing from a monoanionic to a dianionic phosphate group. The isotropic chemical shifts of phosphates in the solid state are similar to those in solution state; very minor differences may well be due to effects on the O-P-O bond angle that are induced on crystallization.

Studies on the PLP cofactor of GPb in solution by NMR have found a similar change in chemical shift on activation of the protein. A shift of 3 ppm downfield was interpreted as resulting from a change from a monoanionic to a dianionic phosphate group when the protein is activated (20, 21). The solid state isotropic chemical shifts of GPb in the two crystalline forms are in the same range as the isotropic chemical shifts for the PLP of GPb in solution, but a smaller difference between chemical shifts for the two crystalline forms is observed than is typical for a change in ionization state. The chemical shift of the tetragonal crystalline form of GPb is nearly identical to the dianionic chemical shift in solution. The chemical shift of the monoclinic crystalline form of GPb is intermediate between that expected for a monoanionic and a dianionic phosphate group.

It has been shown that the principal axes of the ^{31}P chemical shift tensor relate simply to the ground state electron density distribution in the molecule. The principal components of the chemical shift tensor of phosphate groups were found from single crystal studies (39–41, 46) to lie roughly along the P-O bond directions, such that the most downfield component (δ_{11}) lies along the plane of lowest electron density, the most upfield component (δ_{33}) lies along the plane of highest electron density, and the third component (δ_{22}) lies along the bisector of the bond angle of the P-O bonds of highest electron density. A compilation of chemical shift anisotropies (26) and our studies (Taguchi, J. E., S. J. Heyes, and C. M. Dobson, unpublished data) have shown that the components of the principal values of the tensor for monoanionic phosphate monoesters have ppm values in the range of 70 ± 7 for δ_{11}, 6 ± 5 for δ_{22}, and -80 ± 10 for δ_{33}. The principal values of the tensor for dianionic phosphate monoesters showed the components to have values in the range of 70 ± 8 for δ_{11}, -22 ± 7 for δ_{22}, and -31 ± 9 for δ_{33}.

The principal values of the ^{31}P chemical shift tensor used to simulate the spectrum of tetragonal GPb show close similarities to those of the dianionic phosphates presented here and in the literature. In particular the asymmetry parameter, $\eta = 0.42$, is consistent with that (<0.5) expected for a dianion and the anisotropy, $\Delta = \delta_{11} - \delta_{33} = 107$ ppm, is in the middle of the range shown by model dianionic organic phosphate compounds. The δ_{22} values appear to correlate with the formal charge on the phosphate; mono- and dianionic states fall in the particular ranges of 1–11 ppm and −29 to −15 ppm, respectively. The δ_{22} value (−15 ppm) for PLP in tetragonal crystals of GPb, therefore, falls into the range for dianionic phosphates. The principal values of the tensor and the simulation of the MAS spectrum from these values are, like the isotropic chemical shift, effectively identical to those found for dianionic rather than monoanionic phosphate groups in model compounds. As the tetragonal crystal structure has been suggested to be a T state for this form of GPb, since it was obtained in the presence of IMP (10) and shows close similarities to the structure of T state (inactive) glycogen phosphorylase a (47), the observation that the ^{31}P spectrum of PLP in this crystal form is fully consistent with a dianionic state is surprising from the point of view of the solution NMR data. This observation is not, however, inconsistent with the crystal structure; examination of the PLP environment shows contacts to the PLP phosphate from Lys 568 and two peptide NH groups from the helix (shown in Fig. 5 *a*), which would be reasonable for a dianion. Furthermore, a similar conclusion of a dianionic phosphate has been arrived at, independently, by Challoner et al. (48) for crystalline phosphorylase obtained in the presence of glucose under conditions similar to those for which ^{31}P solution NMR has been carried out and the protein shown, in solution, to be in a T state.

The ^{31}P CP/MAS NMR spectrum of the monoclinic crystalline GPb form differs significantly from that of the tetragonal form. Although the principal values of the simulated tensor are closer to those of dianionic than monoanionic model compounds, simulation of the MAS spectrum from a single tensor produces a less satisfying fit than was obtained for the tetragonal form as shown in Fig. 2 *b*. The main differences between the experimental and simulated spectra are in the second order sidebands, which are more intense in the experimental spectrum than in the simulated ones. The possibility of a superposition of sidebands corresponding to two tensors, one for a monoanionic phosphate and one for a dianionic phosphate, was tested by simulating a series of mixtures of the

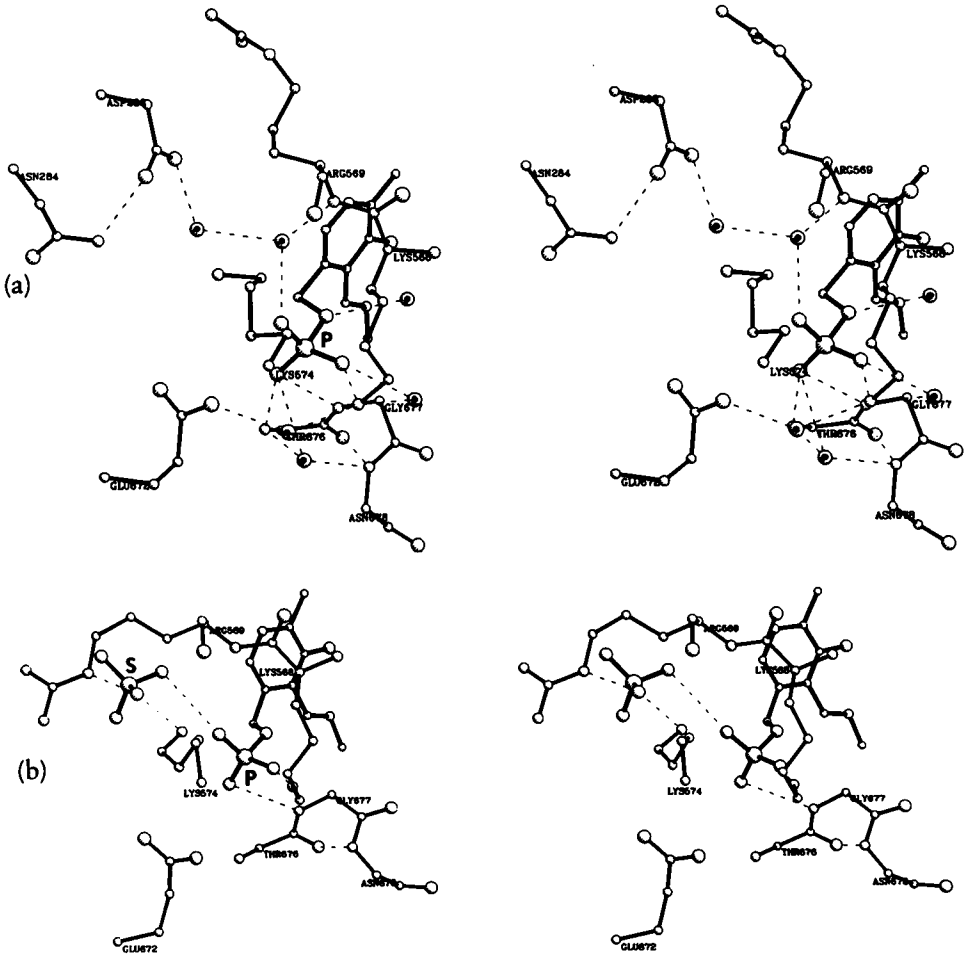

FIGURE 5 Stereo diagrams of the environment of the pyridoxal phosphate 5′-phosphate group in tetragonal and monoclinic crystalline forms of GPb. The phosphate and the sulfate groups are labeled P and S, respectively, in the left-hand image of the stereo diagrams. Water molecules are shown as circles with black dots. (*a*) Tetragonal crystalline form of GPb (T state) (13). The structure has been refined at 1.9 Å resolution which allows for the definite location of water molecules. The cofactor phosphate is solvated with direct contacts to five water molecules, four of which are shown here. The major protein hydrogen bond contacts are from main chain nitrogens of Thr676 and Gly677 at the start of an α helix and from one basic group, NZ Lys568. Each of these groups and water molecules are involved in extensive hydrogen bond networks. One of the phosphate oxygens is hydrogen bonded through two water molecules to the side chain of an acidic residue, Asp283. Two other ionisable groups, Lys574 and Glu672, are in the vicinity but are too far away to make direct contacts to the cofactor phosphate. Main chain atoms only are shown for residues 676–678. Part of the side chain of Lys680 and the glycine loop residues 133–136 have been omitted for clarity. (*b*) Monoclinic crystalline form of GPb (R state) (14). The structure determination at 2.8 Å resolution does not permit definite assignment of waters, and these are not included. The cofactor phosphate makes rather long hydrogen bond contacts to the main chain nitrogens of Thr676 and Gly677. There is a sulfate ion directly hydrogen bonded to the cofactor phosphate. The loop containing Asp283 and Asn284 is displaced, and the side chain of Arg569 has swung down from the T state position to contact the sulfate.

two states using typical tensor values obtained from the model compounds. This procedure resulted in little improvement in the comparison between experimental and calculated spectra and provided no evidence for the existence of a simple physical mix of two ionization states, either from two species of protein or from residual phosphate from the purification of the protein. Furthermore, the possibility of additional phosphate signal arising from residual AMP has been ruled out using absorbance measurements at 260 and 280 nm, which indicated the sample was AMP free. Also, experiments have been carried out on a sample where adenosine 5′-*O*-thiomonophosphate (AMPS) was used in place of AMP in the preparation and purification of GPb. ^{31}P NMR studies of this crystalline sample showed no sign of AMPS present in the sample (unpublished data). AMPS binds more tightly to the GPb than AMP, so the possibility of residual phosphate arising from the presence of the latter is highly unlikely. In the light of the excellent fit of the MAS spectra for tetragonal GPb and the model nucleotides to single tensor interactions, the less good fit for the monoclinic GPb to either a single tensor interaction or a weighted sum of two independent interactions suggests that the shielding of the ^{31}P nucleus in this system cannot be described fully by a single discrete tensor.

The monoclinic crystals are grown in an ammonium sulfate solution; it has been shown that at high concentrations of ammonium sulfate in solution, the protein

may be activated (49). Hence, an R state (active) of GPb is expected and a dianionic PLP expected from solution ^{31}P NMR studies. The possibility exists that local interactions within the protein perturb the tensor significantly from that expected for this ionization state such that it differs from that found for model compounds. There is, however, also a possibility that averaging of the tensor occurs. Some evidence that the latter may be an explanation comes from the observation of the anisotropy, $\Delta = \delta_{11} - \delta_{33} = 96$ ppm, of the "best-fit" tensor. This is significantly less than that typical of dianionic phosphates and considerably less than that for monoanionic phosphates. A possible explanation for the appearance of the monoclinic spectrum is therefore that the phosphate group exists in monoanionic as well as dianionic forms that are in dynamic equilibrium. Dynamic averaging of the phosphate system will tend to reduce the chemical shift anisotropy from the values for the static forms, as observed experimentally, and would be consistent with the δ_{22} value of the best-fit tensor lying between that expected for monoanionic and for dianionic phosphates. If such a dynamic process were to exist and were to be of a rate in the intermediate range of the CSA timescale, then the spectrum would need to be calculated explicitly (50). The resultant sideband manifold intensities will not in general fit to a tensor interaction.

Challoner et al. (48) have concluded that dianionic PLP is present in their crystalline form of GPb, which is expected to be in an R state based on the activity of the protein in solution under conditions used to grow their crystals where AMPS was used as an activator. An examination of the crystal structure for the monoclinic form of GPb (14) shows that a sulfate anion is bound very near (circa 5 Å) to the phosphate group of the PLP cofactor, within H-bonding distance (shown in Fig. 5 *b*). The presence of the sulfate anion could alter the environment of the phosphate group, either influencing the shift tensor directly or perturbing the protonation state of the phosphate. One possibility, for example, is that the effective pK value of the phosphate group could be increased, to become closer to that of the pH of the crystals (7.5). A proton could then be in equilibrium between its site on the phosphate group and a nearby residue or the solvent. Such an equilibrium process could account for the observations by ^{31}P solid state NMR in this work and differences to those observations by Challoner et al. (48).

In conclusion, the solid state ^{31}P NMR experiments described here have enabled the nature of the phosphate group of the PLP cofactor of GPb to be examined under conditions where x-ray structural determinations have been carried out. For the tetragonal (T state) crystals, the phosphate group is demonstrably in a dianionic state. This observation can be rationalized in terms of the crystal structure, as the phosphate makes several contacts with the protein, including direct interaction with a lysine and two main chain NH groups from the start of the α-helix. These contacts to the PLP do not change, although other interactions through water to an aspartate residue are altered during the T to R state transition. In accord with this, despite the presence of sulfate ion in the monoclinic (R state) crystals, the PLP appears also to be predominantly in a dianionic state, although there is evidence for proton exchange between dianionic and monoanionic states in this crystalline form. This suggests that, at least under the conditions used in this study and the crystal structure determinations, the transition between forms of the protein designated as T and R is not accompanied by a simple change in ionization state of the pyridoxal phosphate group.

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