# A solid-state $^{31}$P-NMR investigation of the allosteric transition in glycogen phosphorylase *b*

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ABSTRACT The catalytic role of the cofactor phosphate moiety at the active site of glycogen phosphorylase has been the subject of many investigations including solution-state high-resolution $^{31}$P-NMR studies. In this study the pyridoxal phosphate moiety in both the inactive and active forms of microcrystalline phosphorylase *b* has been investigated by high-resolution $^{31}$P magic-angle spinning NMR. The symmetry of the shielding tensor in model compounds at varying degrees of ionization is investigated and the results indicate a marked difference between the dianionic and monoanionic model compounds. Consequently, the observed similarity in the principal tensor components describing the shielding tensor of the phosphorus nuclei present at the active site of both the R- and T-state conformations suggests that there is no change in ionization state upon activation in contrast to suggestions based upon isotropic shifts. Since previous relaxation measurements have pointed to the need to consider motional influences in such systems, several plausible models are considered. Subject to the assumption of congruency between the principal axis system describing the shielding interaction and a molecular frame determined by the molecular symmetry axes, we conclude that the phosphate cofactor is dianionic in both forms.

## INTRODUCTION

Solid-state $^{31}$P-NMR spectroscopy has wide potential in the study of biological systems and has already provided new insights concerning conformation and internal motion in powder samples of hydrated DNA (Opella et al., 1981; Diverdi and Opella, 1981), oriented DNA fibers (Shindo and Zimmerman, 1980; Nall et al., 1981), enzyme-bound inhibitors (McDermott et al., 1990), phospholipids (Griffin, 1976; Griffin et al., 1978), and other complex biochemicals. In particular, the study of highly oriented DNA fibers by $^{31}$P-NMR spectroscopy has proved a powerful method for deducing the backbone conformation and motions therein, especially under highly hydrated conditions where x-ray diffraction is less useful. The technique also provides a bridge between solution $^{31}$P-NMR data and structures determined by x-ray crystallography.

This investigation concerns a solid-state $^{31}$P-NMR study of the essential pyridoxal phosphate (PLP) cofactor present at the active site of glycogen phosphorylase *b*, a key enzyme in metabolism responsible for the phosphorolysis of glycogen (Fletterick and Sprang, 1979; Johnson et al., 1989). The essential nature of this cofactor, and particularly of its phosphate moiety have been confirmed by studies in which the cofactor has been removed and replaced with various analogues. This has permitted a variety of studies aimed at elucidating the catalytic role of the phosphate moiety, in particular in the determination of its ionization state and its ability to act as an essential proton shuttle during catalysis. Solution $^{31}$P-NMR studies have been widely used to address these questions (Feldmann and Hull, 1977; Withers et al., 1981; Hoerl et al., 1979; Withers et al., 1985). Chemical shifts observed for the enzyme in its inactive (T-state) and active (R-state) conformations suggest that the phosphate moiety exists as a dianion in the active R-state form and as a monoanion in the inactive T-state form, suggesting that the activation of the enzyme (accomplished by binding of the nucleotide activator AMP or its thiophosphate analogue, AMPS) is accompanied by a deprotonation of the coenzyme phosphate. Similar changes were observed as a consequence of the covalent activation of the enzyme via phosphorylation, generating phosphorylase *a*. Activation is accompanied by a high frequency chemical shift change of $\sim$3 ppm, which is essentially identical to the shift change observed upon deprotonation of the free PLP monoanion.

It has been tacitly assumed in most such studies that differences in the $^{31}$P-NMR chemical shifts of the coenzyme phosphate between the T- and R-states are entirely a consequence of the influence of a protonation/deprotonation event. However, this assumption is undoubtedly too simplistic. In the solution state, the isotropic shifts of phosphates are essentially governed by the $\pi$-bond order, the electronegativity of the substituents and the O-P-O bond angles (Lechter and Van Wazer, 1965; Lechter and Van Wazer, 1967; Constella et al., 1976). While the relative importance of such terms has been a question of debate (Gorenstein, 1975; Gorenstein, 1984), it is clear that any geometrical differences that may result on conversion from the T- to the R-state conformation would be expected to influence the chemical shifts. Indeed, a recent analysis of the $^{31}$P shielding tensors of a wide variety of phosphate compounds (Un and Klein, 1989) has established linear correlations between the principal values of the shielding tensor and the O-P-O bond angles and bond lengths. In addition, any changes that result in charge redistribution at the active site might also be expected to lead to a change in the $^{31}$P

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0006-3495/93/02/484/08 $2.00

isotropic shift. Indeed, differences in charge density at the phosphate moiety in the T- and R-states are expected since crystallographic studies of the T- to R-state conversion indicate that the side chain of Arg 569 displaces the side chain of Asp 283 upon activation (Withers et al., 1982; Barford et al., 1988; Johnson et al., 1990). This could either result in deprotonation of the phosphate (if previously protonated) to preserve charge neutrality, or result in chemical shift changes due to the changes in local charge density.

Clearly, without a more precise understanding of the phenomena involved and a quantitative evaluation of the effect on the $^{31}$P-isotropic shift, the information deduced from the solution-state spectra will remain ambiguous. In this study, high resolution $^{31}$P-MASNMR is used to obtain the shielding tensor components of the phosphate moiety of free pyridoxal phosphate in various stages of ionization in the solid state and the results are compared with similar data for the cofactor in both the inactive and active forms of phosphorylase *b*. The principal components describing the shielding tensor interaction allow comparison of the symmetry of the electron density distribution existing at the phosphorus nucleus of the model compounds with that in the two forms of phosphorylase.

Previous studies (Kohler and Klein, 1976; Herzfeld et al., 1978; Tutanjian et al., 1983; McDowell et al., 1988) have established the congruency of the principal axes describing the shielding tensor interaction and the molecular frame, and have been interpreted on the basis that the shielding tensor correlates with the ground state electronic distribution of the phosphate monoester groups. For example, the phosphorus nuclei of the dianionic forms of phosphate monoesters have been shown by both single-crystal studies (McDowell et al., 1988) and static studies of powder samples (Terao et al., 1977) to exhibit axial symmetry, with the unique principal shielding component parallel to the P-O(R) bond. In the case of the monoanionic forms of phosphate monoesters, single-crystal studies of cytidine 5′-monophosphate indicate that the least shielded component of the shielding tensor lies primarily in the RO-P-OH plane containing the two longest P-O bonds, and that the most shielded component of the shielding tensor lies essentially in the plane containing the two shortest P-O bonds where multiple-bond character is expected (Tutanjian et al., 1983). In particular, analysis of single-crystal and powder samples reveals that the shielding tensor symmetry of monoanionic forms of phosphate monoesters is lower than that observed for the dianionic forms. This study assumes that only a minor perturbation of the ground state electronic distribution of the phosphorus nucleus may arise from either geometrical differences between the T- and R-states, or by differences in charge density. Thus, while a shift to high frequency of a particular shielding component by ~9 ppm, or a shift of all the shielding components by ~3 ppm to high frequency might be predicted from either the geometrical or electronic perturbations, the overall symmetry of the shielding tensor is relatively unperturbed. The recent study by Un and Klein (1989), which establishes linear relationships between the various principal shielding tensor elements and O-P-O bond angles and P-O bond lengths, suggests that very modest changes in such parameters can account for a 3-ppm shift of the isotropic resonance. Thus, the pseudo-$C_s$ symmetry existing at the phosphorus nucleus in the case of the monoanion, and the pseudo-$C_{3v}$ symmetry in the case of the dianion are essentially uninfluenced by such changes, ensuring a very different electronic distribution at both nuclear sites (Buckingham and Malm, 1971). The same study suggests that, in general, the symmetry of the shielding tensor is not strongly influenced by the counter-ion.

## EXPERIMENTAL

The PLP monosodium and disodium model compounds were obtained from the free acid by titrating solutions with sodium hydroxide to pH 4.0 and 8.0, respectively, followed by lyophilization. Rabbit muscle glycogen phosphorylase *b* was purified as described previously (Fischer and Krebs, 1962), but using dithiothreitol instead of cysteine, and recrystallised at least three times before use. Adenosine 5′-*O*-monophosphate was removed by dialysis against large volumes of triethanolamine buffer. Crystalline samples of T-state enzyme were obtained by seeding of a solution of phosphorylase *b* (16.7 mg/ml$^{-1}$) in a buffer containing 1 mM EDTA, 5 mM DTT, 10 mM 2-[bis(2-hydroxyethyl)amino]-ethanesulphonic acid, pH 6.7, containing glucose (50 mM), with a highly diluted suspension of seeds obtained by grinding tetragonal crystals of phosphorylase *b* (Johnson et al., 1974; Kasvinsky and Madsen, 1976). Microcrystalline R-state phosphorylase *b* was obtained from solutions of phosphorylase *b* (16 mg/ml$^{-1}$) in a buffer containing 100 mM potassium chloride, 50 mM triethanolamine, 1 mM DTT, 10 mM magnesium acetate, 0.4 mM adenosine 5′-*O*-thiomonophosphate, pH 6.8. The protein microcrystals were packed into the rotor using an Eppendorf centrifuge followed by spinning the rotor in the probe at several kHz for 15 min and removing the residual buffer by pipette. This process was repeated several times if necessary to fill the rotor. Experiments on both the T- and the R-states were initially nonreproducible since the $^{31}$P-MASNMR spectrum sometimes consisted of only isotropic peaks. However, when the above protocol was followed and the samples were spun for prolonged periods before the removal of excess buffer by pipette, a sideband manifold was always observed. Care was taken, however, to ensure that the samples were not completely dried out since many pre-

TABLE 1 **Principal components of the $^{31}$P-shielding tensors for the model compounds of pyridoxal-5′-phosphate in its different phosphate ionization states and of the cofactor phosphate at the active site of phosphorylase *b* in its T- and R-states**

| Compound | $\sigma_{33}$ | $\sigma_{22}$ | $\sigma_{11}$ | $\sigma_{aniso}$ | $\sigma$ | $\delta_{soln}$ |
|---|---|---|---|---|---|---|
| PLP-disodium salt | 79.5 | −14.6 | −43.6 | 108.6 | 7.1 | |
| PLP-monosodium salt | 68.1 | 9.5 | −73.0 | 99.9 | 1.5 | |
| PLP-free acid | 67.2 | 5.5 | −78.7 | 103.8 | −2.0 | |
| R-state phosphorylase *b* | 70.8 | −30.0 | −30.0 | 100.8 | 3.6 | 3.8 |
| T-state phosphorylase *b** | 66.4 | −33.9 | −33.9 | 100.3 | ~−0.5, 2.5 | 0.6 |

All chemical shift values are quoted in ppm. The shifts were referenced to 85% $H_3PO_4$ with the high frequency direction being positive. The anisotropy is defined as $\sigma_{33} - 1/2(\sigma_{22} + \sigma_{11})$. * The shielding tensor components correspond to the peak at −0.5 ppm. Note, however, that a somewhat greater error is introduced into the measured components for this species with respect to the others listed in the table, given the assumptions made when deconvoluting the centerband.

vious x-ray crystallographic experiments have demonstrated the need to keep the crystal "hydrated".

NMR experiments were performed on a Bruker MSL 200 pulse spectrometer with an operating frequency of 81.05 MHz for $^{31}$P and 200.13 MHz for $^{1}$H. A $^{31}$P radio-frequency field of 50 kHz and a proton decoupling field of 62.5 kHz were used in the single pulse, proton decoupled experiments which were the preferred method of experimentation. The program used for simulation of the $^{31}$P-MASNMR spectra incorporated the equations derived by Herzfeld and Berger (1980) using a fast Fourier cosine transformation based on the midpoint rule, and automatic quadrature of a closed type by the Clenshaw-Curtis (Kubo and McDowell, 1990) method for numerical integration. The convention $\sigma_{33} \geq \sigma_{22} \geq \sigma_{11}$ was used for the assignment of the principal tensor components obtained from the spectral simulations of the spinning sideband intensities.

## RESULTS AND DISCUSSION

The principal components of the $^{31}$P shielding tensors of the model compounds were estimated from the singularities of the static spectra and these values were used as a starting point in the direct simulation of the MAS spectra. The results obtained by a least squares fit of the spinning sideband intensities are shown in Table 1. The diprotonated form of PLP was used to complete the series but is not considered a likely ionization state to be observed in the active site of the enzyme. Consideration of Table 1 reveals that the shift of the isotropic resonance, determined by variation in the spinning frequency for a given compound, as a function of protonation is in agreement with the behavior observed in the solution state. Furthermore, the principal components of the shielding tensors describing the phosphorus environment in each of the ionization states are in good agreement with values previously reported in the literature (Duncan, 1990). In particular, the most deshielded component $\sigma_{33}$ is relatively unperturbed by the changes in ionization state, whereas for the dianion, the $\sigma_{22}$ component moves to lower frequency and the most shielded component $\sigma_{11}$ to higher frequency. In the limit of axial symmetry which would be expected for the $^{31}$P shielding tensors of an isolated dianionic phosphate monoester, $\sigma_{22}$ and $\sigma_{11}$ are equivalent and equal to the average of the values typically observed for the monoanion/free acid. In this case the dianionic model compound deviates somewhat from axial symmetry, although it is well known that analysis of the spinning sideband manifold associated with axial symmetry of the shielding tensor is not particularly sensitive (Harris et al., 1988; Clayden et al., 1986). However, the $\sigma_{22}$ and $\sigma_{11}$ components have indeed shifted in the expected directions relative to the monoanion/free acid forms and deviation from axial symmetry may be due to perturbing hydrogen bonding influences with adventitious water molecules. The shielding tensors extracted for the model dianionic compound are, however, in good agreement with those obtained for guanosine 5′-monophosphate (disodium salt, heptahydrate) (Un and Klein, 1989) and various other dianionic phosphomonoesters (Dobson and Taguchi, personal communication) under MAS conditions.

The spectra observed in Fig. 1, *a* and *b*, result from the application of single pulse $^{31}$P-MASNMR experiments to the R- and T-states, respectively, of phosphorylase *b*. Many thousands of transients were required due to the low dilution of the phosphorus nuclei in the crystalline protein sample. For the R-state, an appreciable shielding anisotropy is observed for both the phosphate moiety of PLP and the thiophosphate of AMPS, and the isotropic shifts are similar to those found in solution. Direct simulation of the spectrum arising from the coenzyme phosphate moiety reveals that the best least squares fit is obtained using axially symmetric shielding parameters which are typical of those previously determined for dianionic phosphate monoesters. While the accuracy of the fit may be considered questionable given the presence of only four sidebands in the $^{31}$P-MASNMR spectrum, our simulations demonstrate that in this present case the ratio of the intensity of the centerband to that of the −1 sideband is sensitive to the shielding parameters used in the simulation. This would suggest that the coenzyme phosphate is dianionic in the R-state of the en-

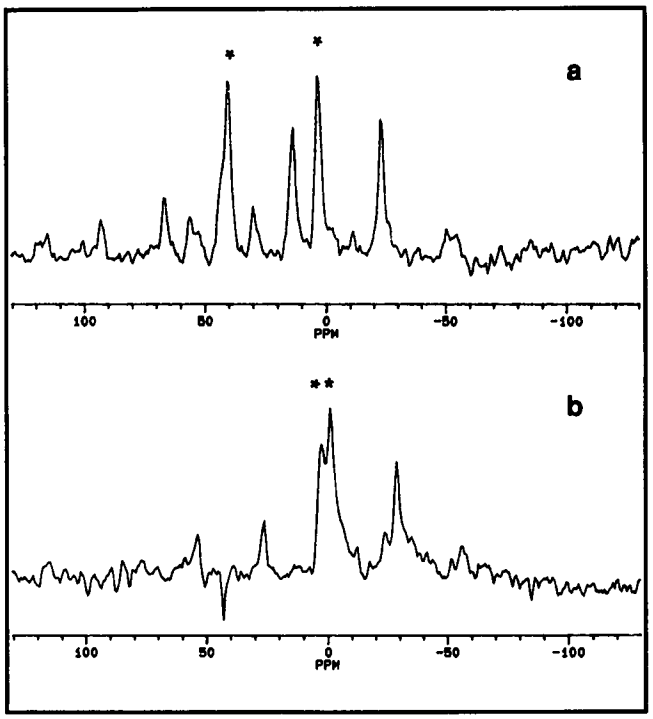


FIGURE 1 Spectra arising from the application of single-pulse $^{31}P$ MASNMR experiments to the study of active and inactive phosphorylase *b* are shown in Fig. 1 *a* and *b*, respectively. An exponential line-broadening factor of 100 was applied for the spectral analysis. Spinning frequencies in the 2.0–2.2 kHz range and a recycle time of 20 s were typically used. The isotropic resonances are indicated on the spectra (*). A total of 29,128 transients were acquired for the active conformer and 11,548 transients in the case of the inactive conformer.

zyme. Such a conclusion, however, makes the assumption that motional averaging does not influence the $^{31}P$ MASNMR spectrum of the phosphate moiety. The possible motional influences may be divided into two categories: (*a*) motion of the microcrystallites themselves facilitated by the presence of intercrystalline water. Previous studies have pointed to the need to examine the crystals in a hydrated environment and the present method of study essentially constitutes a closed system; (*b*) motion of the phosphate moiety at the active site. Recent studies have indicated that the principal axes describing the $^{31}P$ shielding tensor of the phosphate moiety may not be considered to be rigidly fixed in the sample (Withers et al., 1985). In particular, such studies have been interpreted in terms of an increased mobility of the phosphate moiety of the T-state relative to the R-state on the $T_1$ time scale. Although the R-state phosphate moiety is relatively immobilized on the $T_1$ timescale, this does not mean that such a moiety is immobilized in an absolute sense. Indeed, inspection of the three-dimensional structure of phosphorylase by computer graphics confirms the propensity for motion of the phosphate moiety (the most likely and simple mode being rotation of the phosphate moiety about the *P-O(R)* axis). Furthermore, motional influences could also arise in a more subtle manner as a result of exchange of the protons associated with the phosphate moiety with the surrounding lattice. A situation may be envisaged whereby the deprotonation and reprotonation events occur simultaneously so that the net ionization state of the phosphate moiety may be considered constant, although the reprotonation event may involve any of the available phosphate oxygens with equal probability. Such a process may clearly be approximated to a 3-site jump.

The motion in category *a* may be modelled assuming isotropic rotational reorientation characterized by a single correlation time. Under such conditions, the appearance of the observed spectrum is strongly dependent on the frequency of motion relative to the spread of the anisotropic inhomogeneous interaction experienced by a given nucleus in frequency units for a given rotation frequency (Fenzke, 1992). In particular, when the motional frequency exceeds the total shielding anisotropy ($\sim$10 kHz in this present case) the spinning sideband manifold typically observed in the rigid-lattice limit is averaged to a single peak at the isotropic shift of the nucleus. For motional frequencies less than 10 kHz, the same study suggests that motional influences will only be evident from the MASNMR spectrum when the motional frequency exceeds approximately one-tenth of the shift anisotropy. Thus, the observed anisotropy and normal spectral features observed in this case limit any motions to frequencies less than $\sim$1 kHz if, indeed, they exist at all.

For the motion in category *b*, a model assuming anisotropic molecular motion is considered. The presence of such motion under the coherent averaging process of MAS can lead to a potentially complex situation. Motion during the rotor cycle may lead to the destruction of the coherent averaging process of MAS to yield a homogenous lineshape in the sense used by Maricq and Waugh (1979), which serves to complicate the analysis. For simplicity, the assumption is made that the random processes governing the motion are such that the motional frequency greatly exceeds the total shielding anisotropy (rapid anisotropic molecular motion). In such a case, only the secular contributions need to be taken into account and an average Hamiltonian describing the chemical shift interaction may be calculated.

To derive such a Hamiltonian, we consider first of all the transformation from the principal axis system (PAS) describing the shielding tensor interaction to the molecular motion frame (MOL):

$$\mathrm{PAS} \xrightarrow{(\varphi,\theta,\psi)} \mathrm{MOL},$$

where the ($\varphi$, $\theta$, $\psi$) represent the Euler angles for the transformation.

The shielding tensor in the molecular motion frame is related to the shielding tensor described in the principal axis system by the following relationship:

$$\sigma^{\mathrm{MOL}} = R(\varphi, \theta, \psi)\sigma^{\mathrm{PAS}}R^{-1}(\varphi, \theta, \psi),$$

where $R(\varphi, \theta, \psi)$ is the appropriate rotation matrix for the Euler angles $\varphi$, $\theta$ and $\psi$. In particular, the Euler angles $(\varphi, \theta)$ define the transformation aligning $\sigma_{33}$ in the PAS with the $z$-axis of the molecular rotating frame and remain fixed throughout the motional process for the cases of either fast motion about a fixed axis or a 3-site jump. However, the motion induces a time dependence in $\psi$ and for the case of motion about the $z$-axis of the molecular motion frame, it is necessary to average over all the possible $\psi$ to yield:

$$\bar{\sigma}_{33} = \sin^2\theta\cos^2\varphi\sigma_{11} + \sin^2\theta\sin^2\varphi\sigma_{22} + \cos^2\theta\sigma_{33}$$

$$\bar{\sigma}_{22} = \bar{\sigma}_{11} = \tfrac{1}{2}(1 - \cos^2\varphi\sin^2\theta)\sigma_{11} + \tfrac{1}{2}(1 - \sin^2\varphi\sin^2\theta)\sigma_{22} + \tfrac{1}{2}\sin^2\theta\sigma_{33},$$

and the anisotropy, $\bar{\sigma}_{33} - \tfrac{1}{2}(\bar{\sigma}_{22} + \bar{\sigma}_{11})$ is given by:

$$\tfrac{1}{2}(3\cos^2\theta - 1)[\sigma_{33} - \tfrac{1}{2}(\sigma_{11} + \sigma_{22})] + \tfrac{3}{4}(\sigma_{11} - \sigma_{22})\sin^2\theta\cos 2\varphi.$$

These expressions are similar to those derived previously by Mehring et al. (1971). Identical expressions are derived for a 3-site jump model used to simulate proton exchange with the surrounding lattice, in which case $\psi$ is averaged over the relevant discrete angles. Thus, the rapid anisotropic motion effectively defines a new principal axis system which differs from the principal axis system in the absence of motion in the sense that the static principal components of the shielding tensor, $\sigma_{ii}$ $(i = 1, 2, 3)$ are scaled by trigonometrical functions of the Euler angles $(\varphi, \theta, \psi)$. In addition, the "reduced" principal tensor components of the shielding are now aligned differently from the static case with $\bar{\sigma}_{33}$ directed along the $z$-axis of the molecular motion frame. Furthermore, the fast anisotropic motion results in an axially symmetric shielding tensor irrespective of the symmetry of the shielding tensor in the static case.

To derive the Hamiltonian describing the shielding interaction, we consider the total transformation from the frame describing the motionally averaged interaction, MOL, to the laboratory frame (LAB) which may be performed in two steps through the rotor axis system (RAS):

$$\mathrm{MOL} \xrightarrow{(\alpha,\beta,\gamma)} \mathrm{RAS} \xrightarrow{(\omega_r t, \theta_m, 0)} \mathrm{LAB},$$

where $(\alpha, \beta, \gamma)$ are the Euler angles that specify the orientation of MOL for a particular crystallite relative to the RAS, and the Euler angles $(\omega_r t, \theta_m, 0)$ relate RAS and LAB, where $\theta_m$ is the magic angle (54.74°) and $\omega_r$ the spinning frequency.

The relevant spatial part of the interaction in LAB may be expressed as:

$$\hbar^{-1}\bar{H}_{\mathrm{cs}}(t) = \omega_0\{(-1/\sqrt{3})A_{00}^{\mathrm{cs}} + \sqrt{2/3}[A_{20}^{\mathrm{cs}}(t)]\}.$$

Thus, the spatial part consists of both time-dependent and time-independent parts, the time dependence being introduced by sample rotation. The isotropic part, $A_{00}^{\mathrm{cs}}$, is invariant to sample rotation and it is thus necessary to evaluate the spherical tensor components, $A_{20}^{\mathrm{cs}}(t)$, of the shielding tensor which are related to the respective components, $A_{2q}^{\mathrm{cs}}$, in the frame describing the motionally averaged interaction by the mathematical expression:

$$A_{20}^{\mathrm{cs}}(t) = \sum_{qp=-2}^{+2}(A_{2q}^{\mathrm{cs\,mol}})\mathrm{D}_{pq}^{(2)}(\alpha, \beta, \gamma)D_{q0}^{(2)}(\omega_r t, \theta_m, 0),$$

where $D^{(2)}(x, y, z)$ are the elements of the $l = 2$ Wigner rotation matrix. Thus, at the magic angle setting:

$$\hbar^{-1}\bar{H}_{\mathrm{cs}}(t) = \bar{\omega} + \bar{C}_1\cos(\gamma + \omega_r t) + \bar{S}_1\sin(\gamma + \omega_r t) + \bar{C}_2\cos 2(\gamma + \omega_r t) + \bar{S}_2\sin 2(\gamma + \omega_r t),$$

where the coefficients of the dynamic part of the interaction $C_i, S_i$ $(i = 1, 2)$ are analogous to expressions derived by Hezfeld and Berger. For this case of a rotationally averaged axially symmetric shielding tensor in the fast motion limit, the coefficients may be evaluated to yield:

$$\frac{-3\bar{C}_1}{2\sqrt{2}\omega_0} = \sin\beta\cos\beta[\tfrac{3}{4}(\sigma_{11} - \sigma_{22})\sin^2\theta\cos 2\varphi + \tfrac{1}{2}(3\cos^2\theta - 1)\{\sigma_{33} - \tfrac{1}{2}(\sigma_{11} + \sigma_{22})\}],$$

$$\frac{-3\bar{C}_2}{2\omega_0} = \tfrac{1}{2}(\cos^2\beta - 1)[\tfrac{3}{4}(\sigma_{11} - \sigma_{22})\sin^2\theta\cos 2\varphi + \tfrac{1}{2}(3\cos^2\theta - 1)\{\sigma_{33} - \tfrac{1}{2}(\sigma_{11} + \sigma_{22})\}],$$

and

$$\bar{S}_1 = \bar{S}_2 = 0$$

The free-induction decay for the entire sample may then be represented as:

$$g(t) = \frac{1}{4\pi}\sum_N\int \mathrm{d}\Omega I_N\exp[-i(\bar{\omega} + N\omega_r)t],$$

where the intensity of $N$th sideband, $I_N$, is given by $|F_N|^2$

$$\text{and}\quad F_N = \frac{1}{2\pi}\int_0^{2\pi} dX\exp[-i(NX + \Phi(X)]$$

$$\text{with}\quad \Phi(X) = -\left(\frac{\bar{C}_1}{\omega_r}\cos X + \frac{\bar{C}_2}{2\omega_r}\cos 2X\right).$$

Thus, an axially symmetric spinning sideband manifold will always be observed for the influence of fast anisotropic motion of the shielding tensor about a unique axis. However, the sideband manifold will be indepen-

dent of the frequency of motion so long as the motional frequency exceeds the total shielding anisotropy. The possibility thus remains that either the dianionic or monoanionic phosphate moiety may give rise to a spinning sideband manifold characterized by axially symmetric shielding parameters. The shielding parameters obtained by standard analysis of the sideband manifold (in our case, direct simulation) are, however, critically dependent on the Euler angles ($\varphi$, $\theta$). Given that we have defined the most simple (and likely) motion of the phosphate moiety as (or equivalent to) rotation about the *P-O(R)* axis, then evaluation of the angles ($\varphi$, $\theta$) is dependent upon knowledge concerning the relative orientation of the principal axis system of the shielding tensor and the molecular frame. Such information is typically obtained from single crystal measurements which are a clearly inappropriate method for the deduction of the relative orientation of the two frames in this case. With the assumption that the ground state electronic distribution of the phosphorus nucleus determines the orientation of the shielding tensor relative to the molecular frame (i.e., congruency between the shielding principal axis system and a molecular frame determined by the molecular symmetry axes) and a similar geometry of the phosphate moiety present at the active site to that observed in cytidine 5′-monophosphate (Viswamitra et al., 1971), then the Euler angles are given by (90, 37.0), where $\sigma_{22}$ bisects the bond angle describing the two shortest P-O bonds and $\sigma_{33}$ lies in the (R)O-P-O(H) plane. Substitution of these angles and the principal tensor components determined from the analysis of the $^{31}$P-MASNMR spectrum of the model monoanion into the above expression reveals that the reduced axially symmetric pattern that would result from such motion has a significantly lower shielding anisotropy (68 ppm) than that observed for the phosphate moiety present in R-state phosphorylase *b* (100 ppm). The presence of additional motional modes would be expected to lead to still further averaging of the shielding anisotropy. It is therefore reasonable to conclude that, subject to the above assumptions, the R-state phosphate moiety exists as a dianion and not as a highly mobile monoanion. Of course, it is not possible to differentiate between a mobile and a static dianionic moiety using similar assumptions concerning congruency between the principal shielding tensor components and the molecular symmetry axes although this is irrelevant for the purposes of this study. An objection may be that the assumption of the above correlation introduces the possibility of systematic error into the analysis. However, single crystal studies of a wide range of phosphodiesters have indicated that the principal components of the shielding tensor are oriented by at most 15° away from the molecular symmetry axes (Herzfeld et al., 1978; Tutanjian et al., 1983; McDowell et al., 1988). We note, however, that even allowing for a ±20° systematic error in ($\varphi$, $\theta$), our analysis predicts that the observed shielding anisotropy in the R-state still exceeds the maximum theoretical shift anisotropy of a mobile monoanion by the order of 10 ppm thus reinforcing our interpretation.

For the T-state, two sites were observed in the $^{31}$P-MASNMR spectrum, suggesting heterogeneity in the enzyme sample, although there is no evidence for this in the solution state. The (major) lower frequency component has a sideband manifold similar to that observed for the R-state phosphate moiety, but with the isotropic peak shifted to a lower frequency, as seen in solution. Direct simulation of such a sideband manifold leads to axially symmetric parameters with a nearly identical shielding to that observed for the R-state. In this case, however, greater error is introduced by the overlap of the spectral intensity of several components in addition to the uncertainty in the isotropic shift arising from the uncertainty in the phasing of the overlapping centerbands. It is however clear that the shielding tensor symmetry for the major component in the T-state spectrum is very similar to the corresponding phosphate moiety in the R-state spectrum. It would thus seem that the differences that arise between the isotropic shifts of the R- and T-state phosphate moieties are a consequence of the either geometric or electronic influences, and not differences in the protonation state which would be expected to lead to significant differences in the shielding tensor symmetry for the two moieties. Of course, all of the arguments concerning motion of the R-state phosphate moiety apply to the T-state phosphate moiety and we similarly deduce the ionization state of the phosphate moiety in this case to be dianionic. The intensity of the (minor) higher frequency component in the T-state spectrum is too low to permit an objective evaluation of the symmetry of its shielding tensor.

In conclusion, our preliminary results illustrate well the difficulties of obtaining quantitative information from extremely complex biochemical systems in their natural hydrated state via the MAS NMR technique. Furthermore, when the target nucleus is spatially dilute as in the case of the phosphorus nuclei in this study (% wt phosphorus = 0.03), spectral accumulation times become prohibitively long and the further need arises to consider sample stability. In this case comparison of the spectra at different stages in a given MASNMR experiment showed no significant changes as a function of time within the limits of poor signal-to-noise ratio. In spite of these difficulties, our results lead us to the conclusion that the phosphorus nucleus of the cofactor phosphate moiety present at the active site of glycogen phosphorylase *b* in the R-state conformation is characterized by a similar shielding tensor symmetry to that observed for the major component of the spectrum corresponding to the T-state conformation. The origin of the minor phosphate moiety contribution in the T-state spectrum is unknown. Our studies of model compounds suggest that

the symmetry of the shielding tensor of the phosphomonoesters are sensitive to ionization state at least insofar as the differences between the dianionic and monanionic forms are considered, and we conclude that the phosphate moieties in the R- and T-state conformers exist in a similar ionization state in contrast to solution state studies. Our results, however, do not exclude the possibility of a protonation of the cofactor phosphate upon binding of anionic substrates. Furthermore, subject to the assumption that the ground state electronic distribution determines the orientation of the principal axis system of the shielding tensor relative to the molecular frame and the further assumptions concerning the motional modes used, we conclude that the species exhibiting similar shielding tensor symmetry in both the spectra of R- and T-states corresponds to the dianionic form.

Professor R. K. Harris (University of Durham) is gratefully thanked for providing an independent analysis of the sideband spectrum arising from the PLP-disodium salt. Discussions with the group of Dr. C. M. Dobson (University of Oxford) are also acknowledged.

We thank the Natural Sciences and Engineering Research Council of Canada for grants in support of this research.

*Received for publication 21 May 1992 and in final form 8 October 1992.*

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