Frequency-Dependent Phosphorus-31 Nuclear Magnetic Resonance Studies of the Phosphohistidine Residue of Succinyl-CoA Synthetase and the Phosphoserine Residue of Glycogen Phosphorylase *a*†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectra of the active-site N-3 phosphohistidine residue of *Escherichia coli* succinyl-CoA synthetase and the phosphoserine residue on the regulatory site of rabbit muscle glycogen phosphorylase *a* were obtained at four different magnetic field strengths. Plots of observed line width vs. the square of the resonance frequency allowed us to differentiate between relaxation caused by field-independent dipolar terms and field-dependent chemical shift anisotropy terms. From this last contribution we calculated for *E. coli* succinyl-CoA synthetase and for the N-1 phosphohistidine residue of *E. coli* HPr that the phosphorus atom is rigidly held and has no residual mobility on the protein. For the phosphoserine residue of glycogen phosphorylase, we deduced that exchange takes place between two conformations. In general it seems that the phosphorylated residues that are catalytic intermediates in phosphoryl transfer reactions are immobilized, whereas the phosphoserine residues at regulatory sites have some mobility. Moreover, from the difference in the dipolar terms observed for succinyl-CoA synthetase (11 Hz) and glycogen phosphorylase (6 Hz), we deduced that the phosphoryl group of the N-3 phosphohistidine residue is in the monoanionic form and that the phosphoserine residue is in the dianionic form at pH 7.25. This observation is consistent with the pK_as that have been reported for phosphoserine and for phosphohistidine. For both the N-3 and N-1 phosphohistidine residues of proteins, we have calculated an anisotropy term $\Delta\sigma(1 + \eta^2/3)^{1/2} = 230$ ppm. This value is higher than those that we determined from powder spectra of comparable model compounds. Explanations for this discrepancy are considered, including the intriguing possibility that the geometry of phosphorylated intermediates of phosphotransferases is changed to facilitate in-line nucleophilic attack of the phosphoryl group.

Phosphorus-31 nuclear magnetic resonance (^{31}P NMR)[1] has recently become a popular tool for studying the binding of phosphorus-containing ligands to enzymes and other proteins (Cohn & Rao, 1979). In addition this technique allows for noninvasive study of covalently bound coenzymes and phosphoamino acids on proteins. Typical examples of coenzymes that have been studied in this way are FAD on glucose oxidase (James et al., 1980) and some pyridoxal phosphate containing enzymes, most notably glycogen phosphorylase (Feldmann & Hull, 1977; Withers et al., 1981). These studies have provided insight about the environment of the P atoms of these coenzymes. Unfortunately, however, no theory has yet been formulated to translate the observed chemical shifts into molecular details.

Enzymes and other proteins carrying phosphoamino acids can be subdivided into two large groups. The first one is that of the phosphoryl-transferring enzymes, the majority of which catalyze a direct transfer of a phosphoryl group from a phosphate donor (e.g., ATP) onto another substrate. Some phosphotransferases, however, become transiently phosphorylated on a specific amino acid residue; this phosphoenzyme form can thus serve as an intermediate between phosphoryl-donating and -accepting substrates (Knowles, 1980). Amino acid side chains that have been reported to be phosphorylated in this way include those of serine and histidine residues. Examples of enzymes with phosphoserine intermediates that have been studied by ^{31}P NMR are *Escherichia coli* alkaline phosphatase (Bock & Sheard, 1975; Chlebowski et al., 1976; Coleman & Chlebowski, 1979; Hull et al., 1976) and phosphoglucomutase (Ray et al., 1977). The only phosphohistidine-containing protein studied until now is the HPr phosphoryl carrier protein of the bacterial glucose transport system (Gassner et al., 1977; Dooijewaard et al., 1979). This protein is atypical in that it carries the phosphoryl group on the N-1 of the histidine instead of the N-3 where it has been most frequently found (Bridger, 1973). A good representative of this class is *E. coli* succinyl-CoA synthetase (Bridger, 1974).

The second group of phosphoproteins comprises those whose biological activity is regulated by phosphorylation. Unique serine residues usually remote from the active site are phosphorylated and dephosphorylated by specific protein kinases or phosphatases whose activity is under hormonal control (Krebs & Beavo, 1979). Glycogen phosphorylase, for example, becomes markedly activated upon phosphorylation of its serine-14 residue (Fletterick & Madsen, 1980).

It has been demonstrated for a variety of large macromolecular structures that the relaxation of ^{31}P nuclei is dominated by chemical shift anisotropy (CSA), leading to large increases in the observed line width, especially at high field strengths. This effect has been observed for phospholipid vesicles (Berden et al., 1974), for bacteriophages (Opella et al., 1981), and in DNA (Shindo, 1980). Since this mechanism also prevails for relatively small macromolecules such as transfer RNA (Guéron & Shulman, 1975) and actin (Brauer & Sykes, 1981), we were prompted to look for this same phenomenon in phosphoproteins. In this paper we report frequency-dependent ^{31}P NMR studies of the N-3 phosphohistidine residue of *E.*

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[1] Abbreviations: NMR, nuclear magnetic resonance; SCS, succinyl-CoA synthetase; CSA, chemical shift anisotropy; HPr, heat-stable phosphoryl-carrier protein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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coli succinyl-CoA synthetase and the phosphoserine residue of glycogen phosphorylase *a*, leading to the clear demonstration that CSA is the dominant relaxation mechanism at high field strengths. A similar conclusion is reached for the N-1 phosphohistidine residue of HPr. The line widths are further analyzed in terms of the mobility of the protein-bound phosphoryl groups.

Materials and Methods

Succinyl-CoA synthetase was purified from Crooke's strain of *E. coli* grown on succinate-based medium according to methods previously described (Wolodko et al., 1980). Activities were measured and NMR samples were prepared as previously described (Buttlaire et al., 1977). The specific activity was checked before and after NMR spectra were taken and was never below 35 units/mg of protein. NMR samples contained 25 mg/mL enzyme in 40 mM Tris (ultrapure), 80 mM KCl, and 25% D_2O (Bio-Rad), pH 7.2 (meter reading). Phosphorylase was purified from rabbit skeletal muscle and was a gift from Dr. N. B. Madsen and S. Schechosky. Samples were prepared as described by Withers et al. (1981); typically a sample of 35 mg/mL phosphorylase *a* was used in a buffer of 100 mM Tris, 2.5 mM DTT, 1 mM EDTA, and 50% D_2O, pH 7.3. All buffers were passed through a column of Chelex-100 (Bio-Rad) before use.

^{31}P NMR spectra were obtained with four different spectrometers, Bruker WH-400, HXS-270, WH-200, and HFX-90, at the Departments of Chemistry and Biochemistry at the University of Alberta, all operated in the Fourier-transform mode. Normally a 60° pulse angle was applied, and the recycle time for the experiment was 2 s. All spectra of phosphorylase *a* were proton decoupled. The reported line widths are corrected for the line broadening caused by computer digital filtering.[2] Samples of 2 mL were placed in 10-mm Wilmad precision tubes, equipped with Teflon vortex plugs. All experiments were performed at 27 °C. ^{31}P NMR spectra for the powder patterns were obtained at 109.3 MHz without proton decoupling. A pulse angle of 25° was used, and the recycle time was 10 s. The principal elements of the chemical shift tensor were obtained by fitting the observed spectra with calculated powder spectra that were artificially broadened by multiplication with a Gaussian function with full width at half-height of 4200 Hz. The anisotropy values calculated in this way appear to be approximately 15% overestimated in comparison with literature values (see Results and Discussion). This may result from the fact that dipolar couplings were not removed by proton decoupling.

Creatine phosphate, acetyl phosphate, phosphoserine, and cyclic 3′,5′-AMP were obtained from Sigma. Calcium imidazole diphosphate was synthesized according to the method of Rosenberg (1964).

Theory

Relaxation Theory. Several possible relaxation mechanisms can contribute to the nuclear spin relaxation rates of phosphorus nuclei. Since we are concerned here with phosphates covalently linked to proteins, we can assume that chemical exchange does not play a role. Moreover, since spin rotation and scalar relaxation are usually only important for molecules with short correlation times, we will neglect their contributions. Therefore we can confine our calculations here to contributions caused by dipole–dipole interactions between the phosphorus nucleus and neighboring hydrogen and nitrogen nuclei and contributions due to chemical shift anisotropy.

(1) Dipole–Dipole Interactions. The relaxation for a phosphorus atom due to the presence of a hydrogen atom is described (Shindo, 1980; Hull & Sykes, 1975) as

$$\pi\Delta\nu = 1/T_2 = C[4J(0) + J(\omega_P - \omega_H) + 3J(\omega_P) + 6J(\omega_H) + 6J(\omega_H + \omega_P)] \quad (1)$$

where

$$C = \frac{1}{30}S(S+1)\hbar^2\gamma_P^2 r_{PH}^{-6} \quad (2)$$

and the spectral density

$$J(\omega_i) = \frac{2\tau_c}{1 + (\omega_i\tau_c)^2} \quad (3)$$

and where $\Delta\nu$ = line width (hertz), T_2 = spin–spin relaxation time, ω_P and ω_H are the phosphorus and proton frequencies, respectively, γ_i is the gyromagnetic ratio for different nuclei, r_{PH} is the distance between phosphorus and proton nuclei, S is the nuclear spin quantum number, and τ_c is the correlation time. For all proteins and frequencies that we will discuss in this communication, the "nonextreme narrowing limit" condition is fulfilled: $(\omega_P\tau_c)^2$, $(\omega_H\tau_c)^2$, $[(\omega_P - \omega_H)\tau_c]^2$, $[(\omega_P + \omega_H)\tau_c]^2 > 1$. This reduces eq 1 with substitution of eq 2 and 3 to

$$\pi\Delta\nu = \frac{1}{T_2} = \frac{1}{5}\left(\frac{\gamma_P^2\gamma_H^2\hbar^2}{r_{PH}^6}\right)\tau_c \quad (4)$$

Likewise it can be shown that for phosphorus–nitrogen dipole–dipole interaction we find

$$\pi\Delta\nu = \frac{1}{T_2} = \frac{16}{30}\left(\frac{\gamma_P^2\gamma_N^2\hbar^2}{r_{PN}^6}\right)\tau_c \quad (5)$$

(2) Chemical Shift Anisotropy. The expression for the chemical shift anisotropy is (Shindo, 1980; Hull & Sykes, 1975)

$$\pi\Delta\nu = \frac{1}{T_2} = \frac{1}{90}\omega_P^2(\Delta\sigma)^2\left(1 + \frac{\eta^2}{3}\right)[4J(0) + 3J(\omega_P)] \quad (6)$$

For definition of terms, see Hull & Sykes (1975). In the nonextreme narrowing limit we find that

$$\pi\Delta\nu = \frac{1}{T_2} = \frac{4}{45}\omega_P^2(\Delta\sigma)^2\left(1 + \frac{\eta^2}{3}\right)\tau_c \quad (7)$$

It is clear from eq 4 and 7 that the dipole–dipole contributions are not frequency dependent, whereas the chemical shift anisotropy contribution is a function of the square of the magnetic field. The strategy therefore used in this paper is to plot observed line width vs. ω_P^2; the y intercept then gives the line-width contribution from dipole–dipole mechanisms.

Calculation of Overall Rotational Correlation Time for Proteins. For globular proteins the radius of spherical molecules can be directly deduced from the molecular weight: $r = [3M_w\bar{v}/(4\pi N)]^{1/3}$ where r is the radius, M_w is the molecular weight, $\bar{v}$ is the partial specific volume (0.73 mL/g), and N is Avogadro's number. This calculation gives a low estimate for the radius since it neglects contributions due to hydration of the molecule. Typical globular proteins are known to have 0.30–0.40 g of H_2O bound per g of anhydrous protein, leading

[2] To assess contributions of field inhomogeneity, we have measured the line width of a sample of 10 mM inorganic phosphate at all frequencies. The contribution was small and ranged from 0.5 to 0.9 Hz.

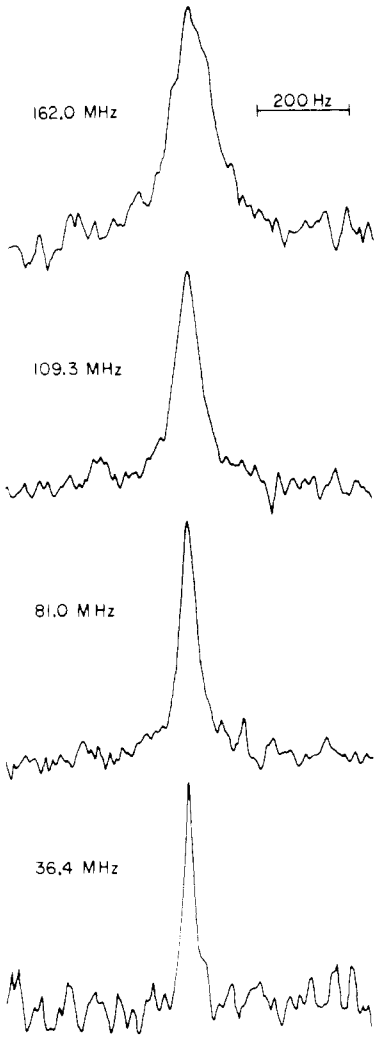

FIGURE 1: ^{31}P NMR spectra measured for a sample of *E. coli* succinyl-CoA synthetase (28 mg/mL, pH 7.2). All spectra were obtained as described under Materials and Methods and are plotted with an instrumental line broadening of 10 Hz. Total number of scans collected for each spectrum was the following: 162 MHz, 27 000 scans; 109.3 MHz, 33 000 scans; 81 MHz, 27 000 scans; 36.4 MHz, 33 000 scans.

to an increase of about 3.0 Å in the radius of each protein [see Cantor & Schimmel (1980)]. For calculations of the overall τ_c, we therefore applied this correction. The overall correlation time can be calculated by $\tau_c = (4\pi\eta r^3)/(3kT)$ where η is the viscosity (0.8513 cP at 27 °C) and T is the temperature (300 K for these experiments). Results for these calculations are shown in Table I. Note that the calculated τ_c's are probably still low estimates since they are based on the assumption that the proteins are round spheres. For different experimental temperatures, different τ_c's have to be calculated, especially since η is strongly temperature dependent.

Results and Discussion

N-3 Phosphohistidine Residue of E. coli Succinyl-CoA Synthetase. This enzyme carries out the substrate-level phosphorylation in the tricarboxylic acid cycle. Both the prokaryotic and the eukaryotic enzymes are known to possess an N-3 phosphohistidine residue that has been established to be an obligatory catalytic intermediate (Bridger, 1974). Here we report on the *E. coli* enzyme which has an $\alpha_2\beta_2$ structure and a molecular weight of 140 000. The two active sites are each arranged at the interface of an α and β subunit, but only one α is phosphorylated at any one time [see Bridger (1974)]. In Figure 1 we show the ^{31}P NMR spectra obtained at four different frequencies. The chemical shift position determined

Table I: Correlation Times Calculated for Several Phosphoproteins[a]

protein	residue	mol wt	calcd radius (Å) hydrated	correlation time (ns)
phosphorylase *a*				
tetramer	P-Ser, PLP	400 000	52	121
dimer	P-Ser, PLP	200 000	42	64
succinyl-CoA synthetase				
E. coli	P-His (N-3)	140 000	37	44
pig heart	P-His (N-3)	70 000	30	23
HPr, *E. coli*	P-His (N-1)	9 000	17	4.2

[a] Calculations were performed as described in the text, for a temperature of 27 °C. Abbreviations: P-Ser, phosphoserine; PLP, pyridoxal phosphate; P-His, phosphohistidine.

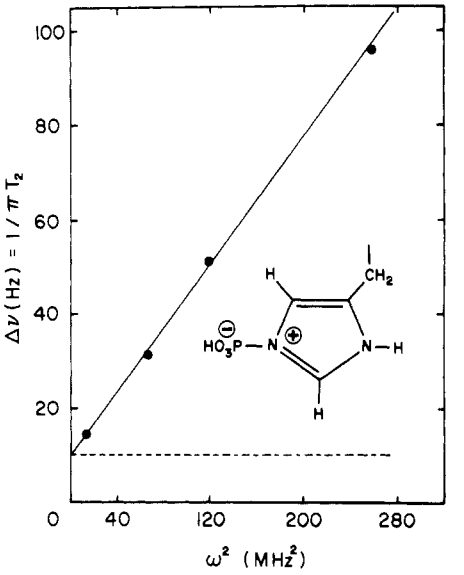

FIGURE 2: ^{31}P NMR line width observed for the N-3 phosphohistidine residue of *E. coli* succinyl-CoA synthetase at four different frequencies. The line widths were measured at half-height, and instrumental line broadening was subtracted. Maximum estimated error per point was 2.5%.

for the resonance is 4.8 ppm upfield from H_3PO_4. This confirms the phosphorylation position of the histidine, since studies of model compounds have shown that N-3 phosphohistidine gives rise to a resonance at 4.5 ppm, whereas the line for N-1 phosphohistidine is at 5.5 ppm (Gassner et al., 1977). Changes observed in the chemical shift and line width of this resonance upon addition of substrates are reported elsewhere (Vogel & Bridger, 1981). The spectra (Figure 1) show that the line width increases markedly with the field strength. This also has a significant influence on the observed signal to noise ratio, which seems optimal at 81.0 MHz. Thus, application of higher frequency instruments for ^{31}P NMR studies on similar proteins will not necessarily lead to better resolution and signal to noise ratio. This behavior is mainly due to the fact that the chemical shift anisotropy becomes the dominant relaxation mechanism at high frequency.

Figure 2 clearly shows this dominance of CSA at higher frequency. For example, at 109.3 MHz, it contributes 40 Hz to the line width which is 75% of the total; at 162 MHz this

Table II: Values of the ^{31}P Chemical Shielding Tensors of Model Compounds[a]

sample	σ_{11}	σ_{22}	σ_{33}	$\Delta\sigma(1 + \eta^2/3)^{1/2}$	ref[b]
imidazole diphosphate (Ca)	125	−55	−70	187[c]	1
creatine phosphate (Na_2)	130	−20	−70	180[c]	1
acetyl phosphate (Li, K)	125	−30	−55	168[c]	1
3′,5′-cAMP (acid)	125	55	−120	219[c]	1
phosphoserine (acid)	75	20	−50	110[c]	1
3′,5′-cAMP	88	38	−124	192	2
phosphoserine	46	3	−52	86	3

[a] All values are in parts per million relative to 85% H_3PO_4. Downfield shifts are given a positive sign. The values are accurate to ±5 ppm. [b] References are (1) this report, (2) Terao et al. (1977), and (3) Kohler & Klein (1976). [c] Calculated with conventions described in Hull & Sykes (1977). These values are probably overestimated by ~15% (see Materials and Methods).

is increased to 90%. Using eq 7 and substituting the observed line width and the correlation time (Table I), we can calculate the anisotropy term $\Delta\sigma(1 + \eta^2/3)^{1/2} = 232$ ppm. This value is quite high; values calculated from the elements of the chemical shift tensor for model compounds seldom exceed 170 ppm. This is discussed in more detail in a subsequent section. If we use eq 7 and solve for τ_c, substituting an anisotropy factor of 187 ppm (see Table II), we obtain a $\tau_c = 84$ ns. Since this exceeds the overall rotational correlation time theoretically calculated for this enzyme (Table I), the phosphoryl moiety is clearly tightly bound and has no mobility on the protein surface. Similar calculations show that the dimeric succinyl-CoA synthetase from pig heart would give a resonance with a total line width of 30 Hz at 109.3 MHz.

The field-independent dipole–dipole contribution can be deduced from Figure 2 to be 11 Hz. In the case of an N-3 phosphohistidine, we have to consider several possible contributions to the dipole–dipole interactions (see insert Figure 2). These are dipolar relaxation due to the two protons on the imidazole ring, protons on the protein that may be hydrogen bonded to the oxygen atoms of the phosphoryl group, a proton covalently attached to one of the oxyanions of the phosphate, or contributions due to the ring nitrogen. These are considered in turn in the following:

(1) The two ring protons are both at a distance of 2.9 Å (Beard & Lenhert, 1968). By substitution into eq 4, using the τ_c as indicated in Table I one obtains $\Delta\nu$(ring protons) = 0.90 Hz.

(2) The hydrogen-bonded protons are presumably at a distance over 2.9 Å (Sundralingam & Putkey, 1970). Since this experiment was carried out in 25% D_2O, this reduces their contributions ($\gamma_D \ll \gamma_H$). We estimate that such protons could maximally contribute 2 Hz to the line width.[3]

(3) A hydrogen atom covalently linked to one of the oxygen atoms of the phosphate would be at a distance of 1.7 Å (Sundralingam & Putkey, 1970). We calculated (25% D_2O) that this contributes 8.2 Hz to the line width.

(4) The distance between the phosphorus and nitrogen atoms is 1.8 Å (Beard & Lenhert, 1968). Using eq 5 we find that this contributes $\Delta\nu = 0.082$ Hz.

[3] Protons of the solvent H_2O would be further away than these hydrogen-bonded protons, and thus contributions to T_2 due to solvent–protein translation motions are negligible.

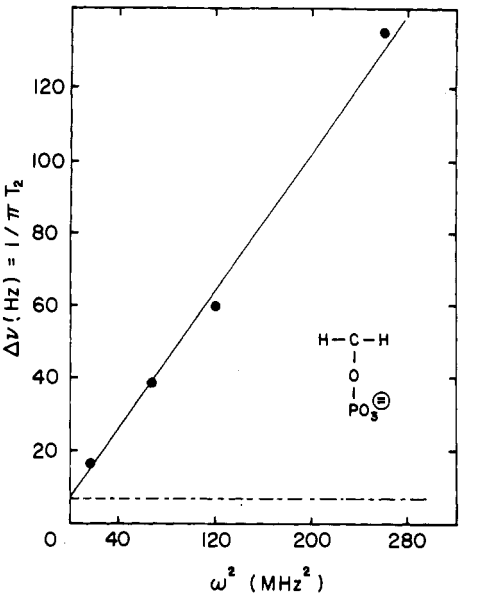

FIGURE 3: ^{31}P NMR line width observed for the phosphoserine residue of glycogen phosphorylase *a* in the presence of 100 mM glucose. Maximum estimated error per point was 6%.

Clearly all contributions to the line width caused by the dipole–dipole relaxation are small, with the exception of the proton on the phosphoryl group which by itself almost fully accounts for the observed field-independent line width. Therefore we propose that the magnitude of the line-width contribution from dipolar interactions indicates that the N-3 phosphohistidine side chain is in the monanion form.

N-1 Phosphohistidine Residue of E. coli HPr. The bacterial phosphoryl carrier protein HPr possesses one resonance in a ^{31}P NMR spectrum at 4.1 ppm upfield from 85% H_3PO_4. This position is characteristic for neither N-3 nor N-1 phosphohistidine model compounds. However, denatured HPr shows a resonance at 5.6 ppm, characteristic of an N-1 phosphohistidine (Gassner et al., 1977; Dooijewaard et al., 1979). At 145.7 MHz, the observed line width of native phospho-HPr is 9 ± 1 Hz. These experiments were performed at pH 7.8 and in 100% D_2O (Dooijewaard et al., 1979), so contributions due to exchangeable protons will be negligible. Other dipolar contributions similar to those considered for succinyl-CoA synthetase can also be neglected due to the shorter τ_c for HPr. We also calculated that the new contribution due to the methylene protons (see insert Figure 2) for the N-1 phosphohistidine is negligible, so the total line width is determined by CSA. We calculated an anisotropy factor of $\Delta\sigma(1 + \eta^2/3)^{1/2} = 228$ ppm. Alternatively, the τ_c calculated for the phosphoryl group using an anisotropy factor as determined for model compounds (Table II) is 11 ns, longer than that determined for the overall correlation time of the protein τ_c. Thus, no residual mobility of the phosphorus moiety on the protein is detected, similar to the result obtained for succinyl-CoA synthetase.

Phosphoserine Residue of Glycogen Phosphorylase. The spectra obtained for this protein showed similar dependence of signal to noise and resolution on field strength as those reported for succinyl-CoA synthetase (data not shown). In the presence of the inhibitor glucose, phosphorylase *a* is in the dimer form, and the ^{31}P resonance observed for the phosphoserine residue does not significantly overlap with that of the pyridoxal phosphate (Withers et al., 1981). Figure 3 clearly shows that in this case the line width at high frequency is also mainly determined by field-dependent mechanisms. Recently, Withers et al. (1981) showed that the thio-

phosphoserine residue of phosphorylase *a* has an almost identical line width for dimer and tetramer, so it seems that the line width of the phosphoserine residue of phosphorylase *a* is more determined by local mobility than by the overall mobility of the protein. From eq 7 we can calculate that the anisotropy term is $\Delta\sigma(1 + \eta^2/3)^{1/2} = 220$ ppm. This clearly is much larger than values found for related model compounds ($\sim$100 ppm). Since in a mobile case this value would have been lower than those for model compounds, the resonance seen is probably an exchange-broadened average of two different conformations with different chemical shifts. Since this chemical shift difference itself is a function of ω^2 (Hull & Sykes, 1975), such a process leads to an even larger increase in line width at higher fields than can be caused by CSA alone. In pH titration experiments (H. J. Vogel and W. A. Bridger, unpublished results), we noted an increase in line width with decreasing pH. Both of these observations support the view that the resulting resonance contains contributions from more than one conformation. Furthermore, Hoerl et al. (1979) reported a split resonance for the phosphoserine residue of glycogen phosphorylase *a*.

The field-independent contribution originating from dipole–dipole mechanisms is 6 Hz, less than that observed for the N-3 phosphohistidine on succinyl-CoA synthetase. This seems surprising at first, considering the longer τ_c calculated for the phosphorylase dimer (Table I). For a calculation of the phosphorus proton dipole–dipole relaxation, we have to consider the two nonexchangeable methylene protons that are at a distance of 2.5 Å (see insert Figure 3) (Sundralingam & Putkey, 1970). Substitution of this distance and a $\tau_c = 64$ ns (Table I) into eq 4 leads to a calculated line width of 3.2 Hz. From X-ray crystallographic data (Fletterick & Madsen, 1980), it is known that the phosphoryl group is surrounded by arginine residues. These contribute hydrogen-bonded protons that will probably contribute up to 3 Hz to the line width (see SCS). These two contributions would account fully for the observed 6 Hz. A covalently linked proton on the phosphoserine at a distance of 1.7 Å (in 50% D_2O) would give rise to an extra 7.7 Hz, so that the total theoretically calculated line width would then be almost 14 Hz, twice that experimentally determined. Moreover, since the observed phosphoserine resonance is probably indicative of two conformations (see above), the experimentally measured value of 6 Hz is an upper limit. From this result and the results of pH titration studies on several protein-bound phosphoserines (H. J. Vogel and W. A. Bridger, unpublished results), we concluded that the phosphoserine is in the dianionic form. This conclusion is also consistent with the complexation by arginine residues detected by X-ray crystallography.

Powder Patterns of Model Compounds. As can be seen from a table of anisotropy factors (Brauer & Sykes, 1981, and references therein) the average values observed for the anisotropy term $\Delta\sigma(1 + \eta^2/3)^{1/2}$ for phosphomonoesters (like phosphoserine) are 120 ppm and those for phosphodiesters are about 170 ppm. Clearly, the values that we calculated for the two phosphohistidines (230 ppm) are larger. However, no results have yet been published for phosphoramidate and acyl phosphate compounds. X-ray crystallographic studies have shown (Beard & Lenhert, 1968) that such compounds deviate from a tetrahedral structure. Hence, large anisotropies can be expected. Table II shows, however, that the values we determined from powder spectra for imidazole diphosphate, creatine phosphate, and acetyl phosphate are quite similar to those observed for phosphodiester compounds. The somewhat larger value found for 3′,5′-cAMP compared to those of other phosphodiesters is presumably caused by the fact that the phosphoryl residue is part of a six-membered ring system. The value determined for phosphoserine is close to those for other phosphomonoesters. However, the values observed for the phosphoramidates clearly do not explain the larger anisotropies found for both protein-bound residues. Similarly, even larger values were reported for ATP bound to actin and phosphoglycerate kinase (Brauer & Sykes, 1981). Possible reasons for this anomaly are considered below:

(1) The simplest explanation is that the estimated τ_c's used for these calculations are too short. We have assumed the proteins to be perfect spheres; deviations from that shape will lead to longer τ_c and hence to smaller anisotropy terms.[4] In fact, when we substituted the anisotropy value 187 ppm (determined from powder spectra of model compounds, Table II), the τ_c's determined for SCS and HPr were 84 and 11 ns, respectively. These values are larger than those calculated theoretically (Table I) and compare favorably to τ_c's determined experimentally by ^{19}F NMR for alkaline phosphatase (Hull & Sykes, 1975) and by fluorescence depolarization measurements for a variety of proteins (Yguerabide et al., 1970). It should be borne in mind, however, that the anisotropy value used to calculate these τ_c's is possibly 15% overestimated when compared to literature values (see Table II). When a 15% lower anisotropy value is used, the calculated τ_c's for SCS and HPr would far exceed the experimental values. We are therefore inclined to the view that uncertainties in the value of τ_c cannot fully account for the larger anisotropy values determined.

(2) As was discussed for phosphorylase *a*, the possibility of exchange between two different bound environments within the intermediate to fast exchange limit cannot always be excluded. This situation can lead to larger observed line widths. The field-dependent contribution is a valid measure of the CSA contribution only when the resonance is that of a single conformation.

(3) It has been proposed that part of the catalytic mechanism of *E. coli* alkaline phosphatase involves the induction of an unusual strain in the bond angles of the phosphoserine intermediate (Bock & Sheard, 1975; Chlebowski et al., 1976). Thus, another possible explanation is that binding to the protein could change the bond geometry of the substituents on the phosphorus atom, which in turn could lead to the larger anisotropy values observed. This is known to cause changes in the chemical shift (Gorenstein, 1975). In fact, the larger CSA value found for the "strained" phosphodiester 3′,5′-cAMP seems to support this view. It is then tempting to speculate that part of the catalytic power of phosphoryl transfer enzymes could involve distortion of the phosphoryl group to a less tetrahedral configuration, thus facilitating the in-line nucleophilic attack that generally occurs (Knowles, 1980).

(4) Changes in electronegativity of the phosphorus substituents caused by the binding to the protein molecule and related changes in the relative amounts of π bonding could lead to the larger anisotropy factors observed, since both can affect electron distribution around the phosphorus atom. These changes could also make the phosphorus atom more electrophilic, thus facilitating nucleophilic attack.

Conclusions

The results presented here for three different phosphoproteins with a range of molecular weights indicate clearly that at high field strength chemical shift anisotropy dominates the

[4] Moreover, possible aggregation in these highly concentrated protein samples could give rise to longer τ_c's.

relaxation rates. Since the T_1 (spin–lattice relaxation time) for this relaxation mechanism is field independent (Shindo, 1980; Brauer & Sykes, 1981), the resolution and the signal to noise ratio will not improve but instead will decrease at higher magnetic fields (see Figure 1). At low magnetic field strength, we find that the relaxation is largely determined by proton–phosphorus dipole–dipole interactions, in agreement with earlier suggestions (Ray et al., 1977). However, Figures 2 and 3 show that even for frequencies as low as 36.4 MHz a considerable portion of the relaxation is caused by field-dependent terms (25% and 55%, respectively). This casts doubt on the validity of calculations that assume domination by either one of the mechanisms at these low frequencies. In contrast, as the frequency is increased, contributions due to dipole–dipole relaxation reduce to less than 10% at 162 MHz.

From a comparison of the data for the N-3 phosphohistidine of *E. coli* succinyl-CoA synthetase and the phosphoserine on rabbit muscle glycogen phosphorylase, we conclude that they are in their monoanion and dianion forms, respectively. This conclusion for the phosphohistidine residue supports the suggestion by Gassner et al. (1977), who propose a pK_a of 8–9, over that of Hultquist et al. (1966), who suggest a much lower pK_a. The cooperative inhibition of succinyl-CoA synthetase by NO_3^- but not by other double negatively charged anions supports this view (H. J. Vogel and W. A. Bridger, unpublished observations).

These studies provide for analysis of line width in terms of functional biochemical properties of three limiting cases of phosphoproteins. These are systems with immobile phosphoryl substituents, those with mobile phosphoryls, and those characterized by more than one conformation:

(1) It is of particular interest that for both active-site phosphohistidines the phosphorus atom appears to be virtually immobilized on the protein. A similar result was reported for the phosphoglucomutase active-site phosphoserine residue (Ray et al., 1977), and the line width reported for metal-saturated alkaline phosphatase (20 Hz at 40.5 MHz; Hull et al., 1976) also implies an immobile residue. T_1 measurements by Chlebowski et al. (1976) have suggested some flexibility for this residue in the apoenzyme, but these workers also reported restriction of motion in metal-saturated enzyme. All of this suggests that immobility of the phosphorylated amino acid residue may be a general property of catalytic intermediates of phosphoryl-transferring enzymes.

(2) In contrast, our studies on regulatory phosphoserine residues have indicated significant mobility. Not only was this observed for the regulatory phosphoserine of glycogen phosphorylase *a* as outlined here but also significant mobility has been detected for similar sites on tropomyosin and ovalbumin,[5] troponin T (Sperling et al., 1979), and myosin light chains (Koppitz et al., 1980). This mobility may be required to facilitate access to the regulatory site by specific protein kinases and phosphatases.

(3) Resonances with larger line width than those deduced from our calculations here have been seen, for example, for the pyridoxal phosphate (form I) of glycogen phosphorylase after addition of the competitive inhibitor α-D-glucopyranosyl 1,2-phosphate (Withers et al., 1981) and for the broadening of the phosphohistidine resonance of succinyl-CoA synthetase in the presence of CoA (Vogel & Bridger, 1981). This extra broadening must be attributed to exchange between at least two conformations with different chemical shifts.

The results obtained here should thus have general applicability to similar studies on phosphoproteins or enzymes with covalently linked coenzymes.

[5] H. J. Vogel and W. A. Bridger, unpublished results.

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Resonance Raman Investigation of Carbon Monoxide Bonding in (Carbon monoxy)hemoglobin and -myoglobin: Detection of Fe–CO Stretching and Fe–C–O Bending Vibrations and Influence of the Quaternary Structure Change†

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ABSTRACT: We report for the first time the direct identification of the iron–carbon bond in (carbon monoxy)hemoglobins (HbCO) and -myoglobin (MbCO) by resonance Raman spectroscopy. The Fe–CO stretching, Fe–C–O bending, and bound C–O stretching vibrations have been detected at 507 (512), 578 (577), and 1951 (1944) cm^{-1}, respectively, in human (carbon monoxy)HbA (sperm whale MbCO) upon excitation at 406.7 nm within the Soret band. These assignments were made on the basis of frequency shifts with the isotopes $^{13}C^{16}O$, $^{12}C^{18}O$, and $^{13}C^{18}O$. Calculated isotope shifts according to the model Im–Fe–C–O (but not Im–Fe–O–C) agree well with the observed data. The possible mechanisms of resonance Raman enhancement of these vibrations are discussed in terms of the $d_\pi(Fe)$–$\pi^*(CO)$ interaction. Careful examination of the Fe–CO stretching mode at 507 cm^{-1} (ρ = 0.055) in both (carbon monoxy)HbA and (carbon monoxy)Hb Kansas with and without inositol hexaphosphate (IHP) reveals no changes in frequency and intensity. This implies that no significant change in the Fe–C bond energy is induced by switching the quaternary structure from the R to the T form in ligated (carbon monoxy)Hb Kansas. The absence of bond tension between the iron atom and the proximal histidine is suggested, as it has been demonstrated that the ν(Fe–CO) frequency is sensitive to a change from 1-methylimidazole to 1,2-dimethylimidazole (as fifth ligand) in model heme–CO complexes. However, the resonance Raman spectrum of carp (carbon monoxy)Hb exhibits a broadening of the Fe–CO stretching mode on the lower energy side upon R → T conversion with IHP, suggesting the presence of a new conformer (or conformers) with a weaker Fe–CO bond or a somewhat different CO distortion.

Carbon monoxide, a competitive inhibitor for oxygen-binding hemoproteins, is a useful probe for heme environment around the distal site. Unlike dioxygen, it is incapable of oxidizing the heme. The bound CO stretching vibration, ν(C–O), can be readily detected by infrared (IR) spectroscopy. Carbon monoxide bound to hemoglobin A (HbA) shows a sharp single absorption band at 1951 cm^{-1} (Alben & Caughey, 1968), whereas in human hemoglobin variants, Hb Zürich (63 E7 His → Arg) exhibits its bound ν(C–O) at 1958 (β) and 1951 (α) cm^{-1}, and (carbon monoxy)HbM Emory (63 E7 His → Tyr) absorbs at 1970 (β) and 1951 (α) cm^{-1} (Caughey et al., 1969; Tucker et al., 1978). Thus, the substitution of distal His E7 by other amino acid residues alters the ν(C–O) frequency in the mutant subunits but not in the normal subunits. More interesting is the observation of multiple ν(C–O) frequencies (1933, 1944, and 1967 cm^{-1}) in the IR spectrum of the monomeric CO complex of sperm whale myoglobin, which was interpreted as indicating three different heme–carbonyl conformers in the same heme cavity (Makinen et al., 1979), although only one conformer has been reported in crystals by neutron diffraction studies (Norvell et al., 1975).

Unlike infrared spectroscopy, detection of axial ligand vibrations by resonance Raman scattering of hemoproteins in dilute aqueous solution is not restricted to the narrow "window" region because water is a weak Raman scatterer (Yu, 1977). In fact, several iron–ligand stretching vibrations such as Fe(II)–O_2, Fe(II)–NO, Fe(III)–OH, Fe(III)–N_3, and Fe(III)–CN have been identified by resonance Raman spectroscopy with the ligand isotope substitution technique (Brunner, 1974; Chottard & Mansuy, 1977; Asher et al., 1977; Tsubaki et al., 1981; M. Tsubaki and N. T. Yu, unpublished results). Moreover, internal ligand vibrations can also be resonance enhanced by tuning the excitation wavelength into a responsible charge-transfer band (Wright et al., 1979; Tsubaki et al., 1981; Yu & Tsubaki, 1980).

Thus, resonance Raman spectroscopy appears to be a powerful technique to study the direct interactions between the heme and its axial ligands. However, its application to (carbon monoxy)hemoglobins or -myoglobins has been limited to pulse laser transient kinetic studies (Woodruff & Farquharson, 1978; Dallinger et al., 1978; Lyons et al., 1978; Coppey et al., 1980; Friedman & Lyons, 1980; Terner et al., 1980), although a preliminary work using continuous wave (CW) laser excitation

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