^{31}P NMR RELAXATION STUDIES OF THE ACTIVATION OF THE COENZYME PHOSPHATE OF GLYCOGEN PHOSPHORYLASE

The Role of Motion of the Bound Phosphate

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ABSTRACT Spin-lattice and spin-spin relaxation rates ($1/T_1$ and $1/T_2$) have been determined for the catalytically essential coenzyme phosphate at the active site of glycogen phosphorylase in both activated (*R* state) and inactive (*T* state) conformations of the enzyme. Dipolar contributions to ^{31}P relaxation due to exchangeable protons on the phosphate group have been determined by measurement of relaxation rates at different concentrations of H_2O and D_2O, and field dependence studies have been performed to estimate the contribution of chemical shift anisotropy to the remaining ^{31}P relaxation in D_2O. At 109 MHz, dipolar relaxation from exchangeable protons was found to account for 50% of the spin-lattice relaxation for activated phosphorylase in 75% H_2O, the remainder being due to chemical shift anisotropy. The spin-lattice relaxation rates in D_2O for *R*-state glycogen phosphorylase are very similar to those measured for other proteins of very different size such as actin (Brauer, M., and B. D. Sykes, 1981, *Biochemistry.* 20:6767–6775), alkaline phosphatase (Coleman, J. E., I. D. Armitage, J. F. Chlebowski, J. D. Otvos, and A. J. M. S. Uiterkamp, 1979), and phosphoglucomutase (Rhyu, G. I., W. J. Ray, Jr., and J. L. Markley, 1984, *Biochemistry.* 23:252–260). In inactive (*T* state) phosphorylase the spin-lattice relaxation rates were almost an order of magnitude slower, while the spin-spin relaxation rates were essentially identical. These results have been analyzed by calculating the theoretically expected ^{31}P relaxation rates in the presence of internal motions that are included in the relaxation calculation using the model-free approach of Lipari and Szabo (1982, *J. Am. Chem. Soc.* 104:4546–4559). The analysis suggests the coenzyme phosphate is relatively immobilized in the activated enzymic conformation, but in the inactive (*T* state) conformation it is considerably more mobile with a rotational correlation time one to two orders of magnitude smaller. Since the spin-lattice relaxation rate for the active *R*-state (immobilized) phosphate is similar to that observed in other phosphoenzymes of different size it is suggested that a librational motion on the nanosecond time scale may constitute a common spin-lattice relaxation pathway for phosphates in macromolecules. The consequences of phosphate motion in terms of recent suggestions concerning the environment and the catalytic role of the coenzyme phosphate are discussed.

INTRODUCTION

The active site of glycogen phosphorylase contains an essential, covalently bound coenzyme, pyridoxal phosphate (PLP)[1]. The location of the coenzyme at the active site was demonstrated by x-ray crystallography (Sygusch et al., 1977; Weber et al., 1978) and it has been shown subsequently that the aromatic ring of the coenzyme is embedded in a predominantly hydrophobic pocket with the phosphate located in an interdomain cleft in close proximity to the active site (Sprang et al., 1982). Recent evidence (Parrish et al., 1977; Withers et al., 1981*b*, Withers et al., 1982*a,b*) has suggested that the phosphates of the coenzyme and of the substrate, glucose-1-P, actually interact during catalysis.

A direct involvement of the phosphate moiety of the coenzyme in catalysis has been demonstrated by means of a variety of analogue replacement studies (Kastenschmidt et al., 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972; Feldmann et al., 1974; Vidgoff et al., 1974; Feldmann and Helmreich, 1976; Parrish et al., 1977; Shimomura and Fukui, 1978; Hoerl et al., 1979; Chang et al., 1983) and in more recent years by ^{31}P NMR investigations (Feldmann

[1] *Abbreviations used in this paper:* PLP, pyridoxal phosphate; PLPP, pyridoxal pyrophosphate; NMR, nuclear magnetic resonance; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time; CSA, chemical shift anisotropy; EDTA, ethylenediamine-tetraacetic acid; DTT, dithiothreitol.

and Hull, 1977; Withers et al., 1981*a,b;* Withers et al., 1982*b*). On the basis of such studies a variety of possible catalytic roles for the coenzyme phosphate have been proposed including potential involvement as an acid catalyst (Pfeuffer et al., 1972; Klein et al., 1982; Klein et al., 1984), a base catalyst (Helmreich and Klein, 1980), a nucleophile (Johnson et al., 1980), or an electrophile (Withers et al., 1981; Takagi et al., 1982; Tagaya and Fukui, 1984). Evidence against the nucleophilic role has been presented by two groups (Takagi et al., 1981; Withers et al., 1982*a*) and further evidence (Withers et al., 1982*c*) has also been forwarded against a role of the coenzyme phosphate as an acid catalyst. In fact this latter study may indeed provide evidence against involvement of the phosphate in any kind of essential proton transfer mechanism. The electrophilic role was proposed on the basis of ^{31}P NMR studies of the coenzyme phosphate of the allosterically activated form of phosphorylase (Withers et al., 1981*a*) and further substantiated (Withers et al., 1981*b*; Takagi et al., 1982; Tagaya and Fukui, 1984) upon the observation of glycosidic bond cleavage in phosphorylase reconstituted with the analogue pyridoxal pyrophosphate glucose, upon addition of oligosaccharide. This electrophilic mechanism requires a distortion of the coenzyme phosphate towards a trigonal bipyramidal configuration to increase the electrophilicity of the phosphorus atom. To gain further insight into the shape and environment of this coenzyme phosphate, a study of its ^{31}P NMR relaxation rates in allosterically active and inactive enzyme was initiated.

A few recent papers (Brauer and Sykes, 1981; Vogel et al., 1982; Coleman et al., 1979; Rhyu et al., 1984) have investigated the longitudinal and transverse relaxation times (T_1 and T_2) for the ^{31}P nuclei of phosphate esters either covalently or noncovalently bound to proteins. Several different relaxation mechanisms have been found to contribute to the overall transverse and longitudinal relaxation rates observed for these nuclei. In the case of ATP bound to G-actin (Brauer and Sykes, 1981), chemical shift anisotropy was found to account for ~90% of the spin-spin relaxation rate at high field and 80% of the spin-lattice relaxation rate, the remainder in each case being due to dipole-dipole relaxation. This assignment was based on frequency dependence studies of the relaxation rates. A similar approach was applied to the phosphohistidine residue in succinyl CoA synthetase (Vogel et al., 1982) where 90% of the spin-spin relaxation at 162 MHz could be accounted for by chemical shift anisotropy. Studies on the phosphoenzyme form of alkaline phosphatase (Coleman et al., 1979) at various concentrations of H_2O and D_2O show that at low field strengths, relaxation is primarily dipolar in nature, and further that >80% of such relaxation is accounted for by dipolar interactions with exchangeable protons. Indeed it was required that such protons be exchanging at a high rate (10^8–10^9 s^{-1}) in order to account for the data. Chemical shift anisotropy contributions would, however, be expected to dominate in this case also at high field strengths. Finally, detailed ^{31}P NMR studies of the phosphoenzyme form of phosphoglucomutase (Rhyu et al., 1984) performed at intermediate field strengths (81 MHz) showed that the spin-lattice relaxation rate ($1/T_1$) for the covalently bound phosphate was dominated by chemical shift anisotropy (90%) in 99% D_2O, but that increasing the H_2O concentration to 90% H_2O increased the dipolar contribution. Similar effects were observed for the spin-spin relaxation rate.

It would therefore appear that dipolar relaxation due to exchangeable protons can be an important relaxation mechanism for phosphates bound to macromolecules, but that chemical shift anisotropy generally dominates at high fields. Similar experiments involving measurements of field-dependence of relaxation rates, and dependence of H_2O concentration, have been performed for the coenzyme phosphate of glycogen phosphorylase in both activated (*R*-state) and inhibited (*T*-state) conformations in an attempt to determine the dominant relaxation mechanisms in each case and thus gain an insight into the role of the coenzyme phosphate and changes occurring therein upon allosteric activation. These studies form the major body of this paper.

MATERIALS AND METHODS

All buffer chemicals and effectors were obtained from Sigma Chemical Co. (St. Louis, MO), except for DTT, which was obtained from Bio-Rad Laboratories (Richmond, CA), D_2O was also from Bio-Rad Laboratories and PLPP was a kind gift of Dr. T. Fukui. pH measurements made in D_2O buffer are uncorrected. Rabbit muscle phosphorylase *b* was prepared as described previously (Withers et al. 1981*a*), and resolved and reconstituted with a twofold excess of PLPP when necessary, according to the published procedure (Withers et al. 1982*a*). The buffer used in all experiments contained triethanolamine (50 mM), potassium chloride (100 mM), EDTA (1 mM), and DTT (1 mM) at pH 6.8 (pH meter reading uncorrected) in D_2O previously treated with Chelex.

^{31}P NMR (109.3 MHz) spectra were recorded as described previously (Withers et al. 1981*a*) on a Bruker HXS-270 spectrometer. Higher field data (162.0 MHz) were recorded on a Bruker WH-400/DS NMR spectrometer (Bruker Spectrospin Ltd, East Milton, Ontario, Canada). T_2 values were determined from the linewidths of the resonances after correction had been made for the exponential line broadening used prior to Fourier transformation. T_1 values were determined by the progressive saturation method (Freeman and Hill, 1971). No ^1H decoupling was used in acquiring any of the ^{31}P NMR data (see Brauer and Sykes, 1981 for discussion of ^1H-^{31}P cross relaxation). Sample size was 1.1 ml in a flat-bottomed 10 mm tube with enzyme concentrations of ~1 mM.

Ultracentrifugation experiments were performed on a Model E analytical ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, CA) at a rotor speed of 56,000 rpm and a temperature of 20 ± 0.1°C. Sedimentation coefficients determined from schlieren patterns were corrected for the viscosity and density of the buffer to water at 20°C.

RESULTS

Glycogen phosphorylase is an allosteric enzyme that can exist in active (*R*-state) or inactive (*T*-state) conforma-

tions in the presence of appropriate allosteric effectors. Glucose and caffeine are allosteric inhibitors that induce the inactive dimeric *T*-state conformation whereas the allosteric activators AMP and AMPS induce the activated tetrameric *R*-state form of the enzyme. The ^{31}P NMR spectra of these enzyme forms have been presented and discussed previously (Withers et al. 1981*a,b;* Feldmann and Hull, 1977; Hoerl et al. 1979). In addition, ^{31}P NMR spectra have been obtained from a form of phosphorylase that is locked into the *R*-state conformation through replacement of the normal coenzyme (PLP) with pyridoxal pyrophosphate (PLPP) (Withers et al. 1982*b*). The resonances that can be observed in these experiments are the phosphate of PLP, which can exist in two forms (form I corresponding to *T*-state and form III corresponding to *R*-state), the α and β phosphates of PLPP, and the slowly exchanging free and bound forms of both AMP and AMPS. Here we present the ^{31}P NMR relaxation time measurements for phosphorylase *b*-PLP plus glucose, caffeine, and AMPS; and phosphorylase *b*-PLPP plus AMP and AMPS (Table I).

Consider first the relaxation time measurements for the tetrameric forms of the enzyme; PLP + AMPS (partial *R* state since no substrate is present), PLPP + AMPS (*R* state), and PLPP + AMP (*R* state). The contribution of the exchangeable protons on the phosphate(s) to the $1/T_1$ relaxation rate was determined by measuring $1/T_1$ vs. the % H_2O in the solvent (see Table I). Since $1/T_2 \gg 1/T_1$, the contribution of the exchangeable protons to the $1/T_2$ relaxation rate is negligible. It can be seen that the exchangeable protons contribute as much as 50% to the observed $1/T_1$ ^{31}P NMR relaxation rate in 75% H_2O at 6.7 Tesla.

It has been demonstrated in several other cases of phosphates bound to macromolecules (Brauer and Sykes, 1981; Vogel et al. 1982; Rhyu et al., 1984) that the dominant ^{31}P NMR relaxation mechanism is chemical shift anisotropy. To be certain this was the case here as well, a minimal study of the field-dependence of the relaxation rates was performed on nucleotide activated PLPP-phosphorylase *b*. Spectra were recorded at 162 MHz and an example is shown in Fig. 1 along with a similar spectrum determined at 109 MHz. The linewidth dependence upon field strength is immediately apparent and is reflected in the values of $1/T_2$ determined, presented with the $1/T_1$ values obtained, in Table I. However, there is essentially no dependence of the spin-lattice relaxation rate upon $1/\omega_0^2$ consistent with CSA dominating $1/T_1$ relaxation (Brauer and Sykes, 1981).

The most interesting results pertain to the change in the relaxation rates upon activation of the enzyme from the *T*-state dimeric form of the enzyme (PLP + glucose or caffeine) to the *R*-state tetrameric forms discussed above. Considering first the spin-lattice relaxation rates ($1/T_1$) measured at 109.29 MHz, a trend towards faster relaxation rates upon activation of the enzyme is quite apparent.

TABLE I
MEASURED RELAXATION RATES FOR PHOSPHATES BOUND TO PHOSPHORYLASE *b*

	Resonance	[H_2O] %	$1/T_1$ (s^{-1})		$1/T_2$ (s^{-1})		
			109.29	162.0	109.29	162.0	
			MHz	*MHz*	*MHz*	*MHz*	
PLP + Glucose	PLP	~5%	0.033 (−1.48)*		250 (2.40)*		Dimer
PLP + Caffeine	PLP	~5%	0.031 (−1.51)*		250 (2.40)*		Dimer
PLP + AMPS	PLP	~5%	0.15 (−0.82)*		500 (2.70)*		Tetramer
	PLP	0	0.17 (−0.77)*		500		
		75	0.34		400		↓
	AMPS Free	0	0.12		160		
		75	0.17		140		
	AMPS Bound	0	0.13		440		
		75	0.16		300		
PLPP + AMP	PLPPα	~5%	0.37	0.43	650	1600	Tetramer
	PLPPβ		0.36	0.38	450	1100	
							↓
PLPP + AMPS	PLPPα	~5%	0.37		660		
	PLPPβ		0.40		440		

*Values in parentheses are log (relaxation rate).

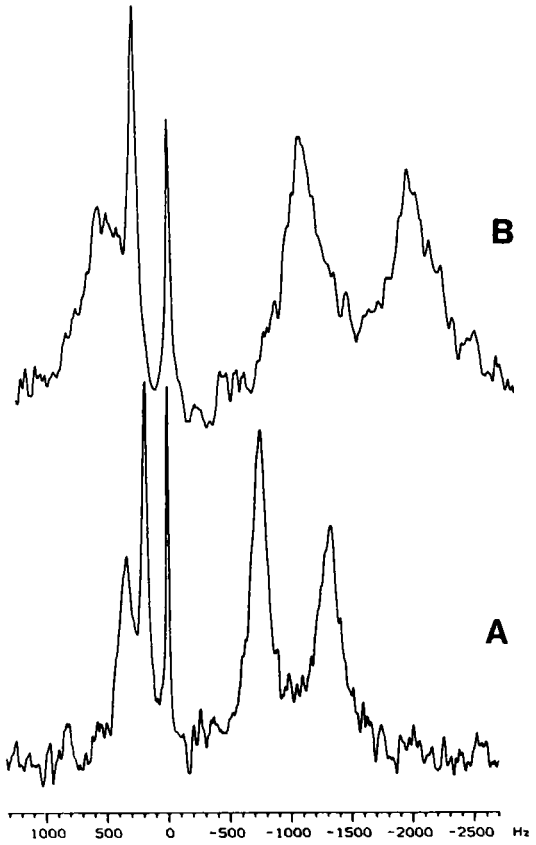

FIGURE 1 ^{31}P NMR spectra of PLPP-phosphorylase *b* (1.09 mM) plus AMP (1.92 mM), pH 6.8, 28°. (*A*) 109.29 MHz spectrum, sweepwidth ±2,500 Hz, 2,048 points, 90° pulse, 7 s between acquisitions, 5,000 acquisitions; (*B*) 162 MHz spectrum, sweepwidth = 10,000 Hz, 2,048 points, 90° pulse, 7 s between acquisitions, 5,000 acquisitions.

Indeed, since the nonextreme narrowing limit is expected to apply in all cases discussed, the observed relaxation rate will be inversely proportional to the rotational correlation time (Brauer and Sykes, 1981). Consequently, the relaxation rates observed for the tetrameric (activated) species should be half the value that would be observed in a dimer of the same conformation. Therefore the fivefold increase in spin-lattice relaxation rate observed for the PLP phosphate upon addition of nucleotide activator should, in reality, be interpreted as a 10-fold increase taking this factor into account. Similarly, the spin-lattice relaxation rates for the fully activated *r*-state PLPP-phosphorylase *b* can be considered as 20 times greater than that for the PLP phosphate of *T*-state phosphorylase *b*, as far as the environment of the phosphate is concerned, rather than the 10 times greater that is experimentally observed.

The spin-spin relaxation rates also increase upon activation of the enzyme structure. However, in the nonextreme narrowing limit as discussed above, it is expected that the spin-spin relaxation rate will be directly proportional to the rotational correlation time. Therefore the twofold increase observed in $1/T_2$ upon nucleotide activation is due solely to the doubling of the rotational correlation time as a consequence of tetramer formation. This implies that there is no intrinsic increase in linewidth for the phosphate itself beyond that expected from the change in overall molecular weight of the enzyme solution.

Since the oligomeric states assigned to these enzyme-effector complexes are based upon published values determined at enzyme concentrations of 5 mg/ml, the quaternary organization of each was investigated by ultracentrifugal analysis at higher enzyme concentrations of up to 58 mg/ml as described in Materials and Methods. No evidence was obtained for an increase in sedimentation constants with sample concentration, which would be consistent with tetramerization in any system that existed as a dimer at low concentrations. Indeed, in the dimeric system most likely to tetramerize at high concentrations, i.e., unliganded phosphorylase *b*, a slight decrease in $S_{20,w}$ value was observed due to the increasing viscosity of the solution. Unfortunately, it was not technically feasible to extend these measurements to concentrations of 100 mg/ml as actually used in these NMR experiments. However, as similar linewidths have been measured for the ^{31}P NMR resonance of the PLP phosphate in phosphorylase at concentrations of 50 mg/ml and 100 mg/ml (Withers et al. 1979; 1981), it is assumed that the oligomeric state does not change with concentration in this range.

DISCUSSION

The results of the relaxation measurements presented in this paper demonstrate: (*a*) that exchangeable protons can significantly increase the relaxation rate of macromolecule-bound phosphate nuclei, (*b*) that the remaining relaxation of macromolecule-bound phosphates is dominated by chemical shift anisotropy relaxation, and (*c*) that there is a very large change in the spin-lattice relaxation of the PLP phosphate of glycogen phosphorylase *b* upon activation from the *T* to the *R* state.

That the exchangeable protons contribute significantly to the relaxation of the enzyme-bound phosphates is important to recognize in relaxation studies (Rhyu et al., 1984) and has been demonstrated and considered theoretically by several other workers (Coleman et al., 1979; Vogel et al., 1982). For very large enzymes where $1/T_1$ becomes very small because of the increase in τ_c this also has a very practical consequence. Since very small $1/T_1$ relaxation rates will limit the efficiency of acquisition of spectra in the Fourier transform mode, the amount of D_2O added for a lock signal should be kept to the minimum required for the lock if relaxation measurements are not of interest.

The observation that the relaxation of the PLP phosphate of tetrameric *R*-state phosphorylase species is dominated by the contribution of CSA to the relaxation is also not unexpected in light of other work (Brauer and Sykes, 1981; Vogel et al., 1982; Rhyu et al., 1984). Considering

an immobile phosphate on an enzyme of sufficient size such that the NMR relaxation is outside of the extreme narrowing limit $[(\omega_o\tau_c)^2 \gg 1]$, the relationship between $\Delta\upsilon_{CSA}$ and $(1/T_1)_{CSA}$ is given by

$$\Delta\upsilon_{CSA} = (1/T_1)_{CSA}\frac{2\,\omega_o^2\tau_c^2}{3\pi},$$

where $\Delta\upsilon_{CSA}$ and $1/T_1)_{CSA}$ are the contributions of CSA relaxation to the linewidth and spin-lattice relaxation rate, respectively; ω_o is the larmor frequency for ^{31}P, and τ_c is the rotional correlation time. At 109 MHz for ^{31}P NMR, and $\tau_c = 121 \times 10^{-9}$ s for the tetrameric form of phosphorylase (Vogel et al., 1982) this becomes

$$\Delta\upsilon_{CSA} \cong 1{,}500\,(1/T_1)_{CSA}.$$

Experimentally, for PLPP + AMPS one obtains $\Delta\upsilon/(1/T_1) = 2{,}100$ for the α phosphate and 1,550 for the β phosphate. This is certainly in reasonable agreement with the expected result if CSA is the dominant mechanism and provides a plausible explanation of the relaxation in light of the assumptions and experimental errors involved.

The above explanation, however, does not allow us to understand the change in the relaxation between the *T*- and *R*-state forms of the enzyme, which involves the expected change in $\Delta\upsilon$ with change in molecular weight, but a 10-fold increase in $1/T_1$ upon going to the *R* state after correcting for the change in molecular weight. This result suggests that some fast correlation time process that could influence $1/T_1$ but not be seen in $1/T_2$ (which is dominated by the spectral density, $J(0)$) is changing between the *T*- and *R*-state forms.

Further, we have recognized that the $1/T_1$ relaxation rates of the tetrameric form monophosphates are all near $1/T_1 \sim 0.12$–$0.17\ s^{-1}$ when the contribution of exchangeable protons is removed. This is essentially the same number obtained by a number of other workers for many other proteins of very different molecular weights; alkaline phosphatase ($1/T_1 = 0.12\ s^{-1}$; (Coleman et al., 1979); actin ($1/T_1 = 0.19$–$0.20\ s^{-1}$; (Brauer and Sykes, 1982); and phosphoglucomutase ($1/T_1 = 0.13\ s^{-1}$; (Rhyu et al., 1984) being examples. This suggests that $1/T_1$ in all of the systems is dominated by some universal fast relaxation time process ($\tau_e \sim 1/\omega_o$). The obvious candidate is some form of fast internal motion of the phosphate.

We feel we can rationalize and understand the above-mentioned results in terms of a model for the relaxation that incorporates internal motion. Rather than specify the exact nature of the motion (rotation, libration, etc.) we have chosen to use the model-free approach of Lipari and Szabo (1982) for the calculation of the relaxation rates. Further, while we will only calculate the CSA contribution to the relaxation, we emphasize that other contributions such as dipolar interactions with the CH_2 protons would have a very similar dependence on internal motions. Taking $(1/T_1)_{CSA}$ and $(1/T_2)_{CSA}$ as

$$(1/T_2)_{CSA} = (2/90)\,\omega_o^2(\Delta\sigma)^2(1 + \eta^2/3)\{4J(0) + 3J(\omega_o)\}$$

$$(1/T_1)_{CSA} = (2/15)\,\omega_o^2(\Delta\sigma)^2(1 + \eta^2/3)\{J(\omega_o)\},$$

with the spectral density given by

$$J(\omega) = S^2\{\tau_c/[1 + (\omega\tau_c)^2]\} + (1 - S^2)\{\tau/[1 + (\omega\tau)^2]\},$$

where $1/\tau = 1/\tau_e + 1/\tau_c$; where τ_c is the overall rotational correlation time of the enzyme molecule, τ_e is an effective correlation time that depends on the rate and detailed nature of the internal motion, S^2 is the "order parameter" corresponding to the internal motion (a measure of the degree of spatial averaging of the motion) and $(\Delta\sigma)(1 + \eta^2/3)^{1/2}$ is the chemical shift anisotropy of the phosphate nucleus. For the purpose of this calculation we have chosen $\tau_c = 200 \times 10^{-9}$ s for the tetrameric form and 100×10^{-9} s for the dimeric form, and $(\Delta\sigma)(1 + \eta^2/3)^{1/2} = 240 \times 10^{-6}$ (Brauer and Sykes, 1981). Presented in Fig. 2 are the calculations of $1/T_1$ and $1/T_2$ for both forms of the enzyme for various values of S^2 as a function of τ_e. Within this discussion we are trying to rationalize the relaxation behavior with reasonable values for the parameters, not to calculate it exactly. Somewhat more emphasis is placed on the T_1 measurements because of other processes such as chemical exchange that can influence the line-width measurements.

In Fig. 2 we can see the following. First the spin-spin relaxation rates $(1/T_2)$ and consequently the linewidths $(\Delta\upsilon = 1/\pi\ T_2)$ are generally larger and the spin-lattice relaxation rates $(1/T_1)$ generally are smaller in Fig. 2 *B* with respect to Fig. 2 *A* because of the difference in overall correlation time between dimer and tetramer. An exception to this trend is the value of $1/T_1$ in the presence of internal motion with correlation time $\tau_e \sim 1/\omega_o$. Under this circumstance, $1/T_1$ is relatively independent of the overall correlation time τ_c. Second, the linewidths are close to the experimentally measured values. Third, the presence of internal motion does not dramatically influence the linewidths because of the large τ_c's involved. Fourth, if no internal motion is present $1/T_1$ cannot be fit for either the dimer or the tetramer; T_1 is never as long as is measured for the dimer or as short as measured for the tetramer. Only when there is some, however small (i.e., $S^2 = 0.8$), amount of internal motion near the Larmor frequency do the calculated T_1's approach the experimentally measured values for the tetramer; and only in the presence of rapid large scale internal motions do the calculated T_1's approach the measured values for the dimer. Thus, inclusion of internal motion explains the differences observed between the *T*- and *R* state forms of phosphorylase, and provides a rationale for the observed lack of dependence of

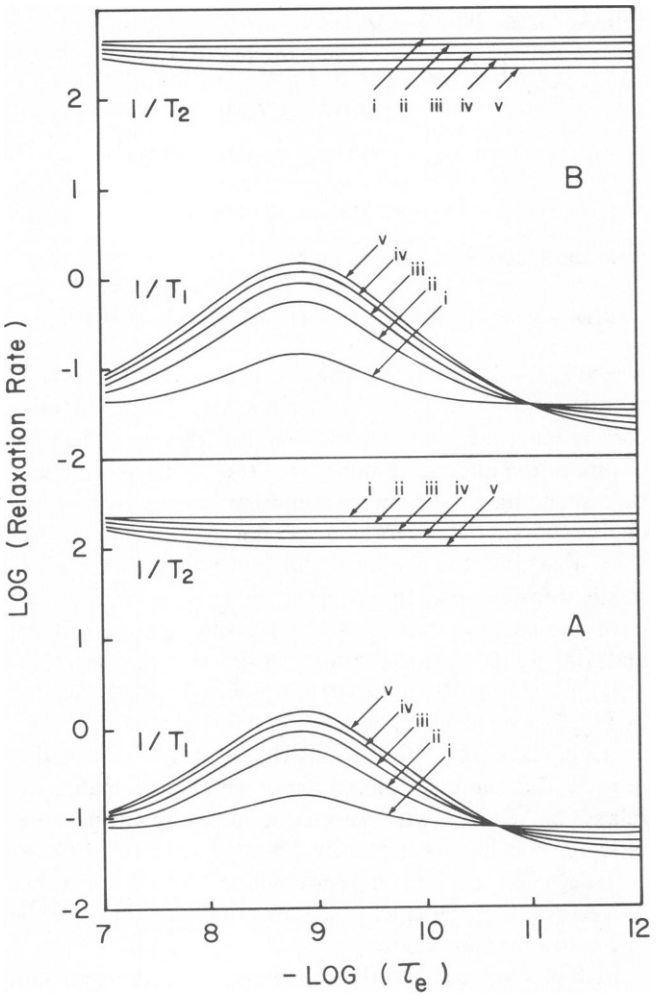

FIGURE 2 Calculated values of $1/T_1$ and $1/T_2$ for ^{31}P NMR of a phosphate group attached to glycogen phosphorylase *b* assuming chemical shift anisotropy as the dominant relaxation process, and including internal motions using the model free approach of Lipari and Szabo (1982). Calculations were done for a ^{31}P NMR frequency of 109.29 MHz, assuming an anistropy $\Delta\sigma\ (1 + \eta^2/3)^{1/2}$ of 240 ppm, for an overall correlation time τ of 1×10^{-7}s (panel *A* shows dimeric phosphorylase *b*) and 2×10^{-7}s (panel *B* shows tetrameric phosphorylase *b*). The top set of curves in both panels are for $1/T_2$, the bottom set of curves on both panels for $1/T_1$. For each set of curves labeled *i* to *v*, the values of S^2 (see text for definition) were (*i*) 0.96, (*ii*) 0.81, (*iii*) 0.67, (*iv*) 0.55, and (*v*) 0.44. The plot for $S^2 = 1$ is a horizontal line running through the intersection point, for $1/T_1$ and essentially identical to plot (*i*) for $1/T_2$.

$1/T_1$ relaxation rates on molecular weight. The $1/T_1$ values for tetrameric *R*-state phosphorylase can be "fit" in terms of the parameters and calculations presented by values of S^2 near 0.8, and τ_e near 10^{-9} s for example, although quite a range of values of S^2 and τ_e are formally possible. The $1/T_1$ values for dimeric *T*-state phosphorylase can only be "fit" for small values of S^2 and values of τ_e near 10^{-11} s, that is, with one to two orders of magnitude faster motion in the *T*-state than the *R*-state. The dramatic change measured in the spin-lattice relaxation rate can therefore be accounted for if the coenzyme phosphate is more loosely restricted (i.e. more mobile) in the *T*-state and more tightly restricted in the *R*-state. The fact that the $1/T_1$ relaxation rate is relatively insensitive to the overall correlation time for this value of τ_e suggests that some "universal" fast internal motion might dominate the spin-lattice relaxation of macromolecular-bound phosphates on a variety of enzymes of very different molecular weights.

Since the values of $1/T_1$ might be expected to be sensitive to the spectrometer frequency at which they were measured, these calculations were also performed for a spectrometer frequency of 162.0 MHz (results not shown). The calculated values of $1/T_2$ increase dramatically at 162.0 MHz for all values of τ_e (proportional to ω_o^2) as observed experimentally. Less change is observed in the calculated curves for $1/T_1$. For values of $\tau_e > 10^{-8}$ s, the calculated values of $1/T_1$ are essentially equal to those calculated at 109 MHz. However, the position of the maximum in these curves is shifted on the $-\log(\tau_e)$ axis from 8.8 to 9.0, and the relaxation rates are slightly higher at the maximum (<40%). The higher frequency internal motion therefore appears to effectively decouple $1/T_1$ from dependence on spectrometer frequency, especially at longer τ_e's. This is consistent with the observed apparent invariance of $1/T_1$ for PLPP as measured at the two frequencies (Table I), although these T_1's are the shortest of all so that other contributions may be masking any effect. This is also interesting in regard to measurement of similar $1/T_1$ values for several phosphoenzymes of different molecular weight, since these relaxation rates were also measured at different spectrometer frequencies.

^{31}P NMR studies of the catalytically essential coenzyme phosphate of glycogen phosphorylase have been performed previously (Feldmann and Hull, 1977; Hoerl et al., 1979; Withers et al. 1979; 1981*a*, *b*; 1982*a*, *b*, *c*; Klein et al., 1984; Withers, 1985) and various predictions made as to the state of the phosphate in the presence of various allosteric effectors on the basis of observed chemical shifts. It was suggested that in the presence of the allosteric inhibitors glucose and caffeine, the coenzyme phosphate is monoanionic, but that upon addition of the allosteric activator AMPS the phosphate deprotonates and becomes dianionic. Later experiments (Withers et al. 1981*a*) performed in the presence of both nucleotide and a substrate analogue led to two possible interpretations regarding the coenzyme phosphate. Either it is monoprotonated with its protons in an intermediate exchange regime, or dianionic but tightly coordinated with and constrained by adjacent positively charged amino acid sidechains. The observations reported in this paper are quite consistent with these general interpretations and may suggest a likely answer to the uncertainties in assignment as follows. A monoprotonated (monanionic) phosphate as proposed for *T*-state phosphorylase would indeed be expected to be less tightly coordinated and constrained than a dianionic phosphate as in the *R*-state enzyme since it has only one negative charge available for electrostatic interaction with adjacent amino acid sidechains and therefore a lower available interaction

energy. Therefore it would appear that decreasing mobility upon activation (as observed here) would be quite consistent with deprotonation of the coenzyme phosphate (as deduced previously). Taking this one step further if addition of AMPS, which induces a partial *R*-state enzyme, results in deprotonation and partial immobilization it would not seem unreasonable to suggest that further activation towards a full *R* state (by addition of a substrate analogue) would result in further immobilization and possibly distortion, of the coenzyme phosphate. The higher relaxation rates observed for PLPP-phosphorylase, a covalently activated form, would seem to bear this out. This would of course be consistent with only one of the possible interpretations of the data in that experiment and also consistent with, but by no means proof of, recent proposals (Withers et al., 1981*b*, and 1982*b*; Takagi et al., 1982; Tagaya and Fukui, 1984; Withers, 1985) concerning the catalytic role of the coenzyme phosphate as an electrophile. Unfortunately, it is not possible at present to test this directly by measuring the spin-lattice relaxation rates for phosphorylase and both nucleotide and substrate analogue since the latter is not sufficiently stable to survive the length of experiment necessary without significant decomposition to phosphomonoesters, which obscure the resonance from the coenzyme phosphate.

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