^{31}P NMR Studies of Enzyme-Bound Substrate Complexes of Yeast 3-Phosphoglycerate Kinase. 2. Structure Measurements Using Paramagnetic Relaxation Effects of Mn(II) and Co(II)†

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ABSTRACT: Measurements of the paramagnetic effects of two dissimilar activating paramagnetic cations, Mn(II) and Co(II), on the spin relaxation rates of the ^{31}P nuclei in the complexes of 3-phosphoglycerate kinase with ATP, ADP, and 3-P-glycerate have been used to study the structures of these enzyme–substrate complexes. All experiments were performed on enzyme-bound complexes, so that two exchanging complexes (with and without cation) contribute to the observed relaxation rate. Measurements were made at three ^{31}P NMR frequencies, 81, 121.5, and 190.2 MHz, and as a function of temperature in the range 5–20 °C to determine the effect of exchange on the observed relaxation rates. Relaxation rates in E·MnADP and E·MnATP were shown to be exchange-limited, and therefore bereft of structural information, both by lack of frequency dependence and by temperature dependence with activation energies (ΔE) in the range 5–8 kcal/mol. Relaxation rates for E·CoADP and E·CoATP exhibit frequency dependence and ΔE values in the range 1–3 kcal/mol; i.e., these rates depend on the Co(II)–^{31}P distances. Difficulties involved in estimating electron relaxation times in E·CoADP and E·CoATP restrict calculation of Co(II)–^{31}P distances in these complexes to upper and lower limits. These distances were all in the range 2.7–4.1 Å, appropriate for direct coordination of Co(II) to the phosphate groups. In the quaternary complex E·MnADP·3-P-glycerate, although the ^{31}P relaxation rates of α-P and β-P (ADP) were exchange-limited, that for 3-P (3-P-glycerate) was not exchanged-limited [because of the longer Mn(II)–^{31}P distance], as evidenced by its frequency dependence and an activation energy of 1.8 kcal/mol. The frequency dependence of the relaxation times of 3-P-glycerate was used to determine a Mn(II)–^{31}P (3-P-glycerate) distance of 11.1 ± 0.3 Å, suggesting that in E·MnADP·3-P-glycerate the enzyme is in an open conformation.

A decisive step in the quest for a molecular basis of enzymatic catalysis is the acquisition of reliable structural information on enzyme-bound substrate complexes. For enzymes in which paramagnetic cations are present as prosthetic elements and for those in which such cations can be substituted as activators in reaction complexes [e.g., Mn(II) and Co(II) for ATP[1]-utilizing enzymes] measurement of spin relaxation rates of nuclei in the presence of the cations offers an attractive method for obtaining structural information in solution. The paramagnetic cation measurably enhances relaxation rates of nuclei in its vicinity in a manner proportional to the reciprocal sixth power of the cation–nucleus distance. However, early applications of this method to position the cations in the ATP complexes of pyruvate kinase (Mildvan et al., 1976) and phosphoribosylpyrophosphate synthetase (Granot et al., 1980) led to results that disagreed with those of other methods such as ^{17}O superhyperfine structure effects in Mn(II) EPR spectra (Lodato & Reed, 1987) and metal ion effects on the phosphorothioate analogues of nucleotides (Gibson & Switzer, 1980).

Recent work on the structure of metal–nucleotide complexes of creatine kinase based on ^{31}P NMR measurements in the presence of Mn(II) and Co(II) (Jarori et al., 1985) showed that (i) the cation is directly coordinated to all three phosphate groups of ATP on this enzyme; (ii) the role of exchange is, in general, incorrectly assessed in previously published NMR measurements and is the most likely cause for the aforementioned discrepancies; (iii) the contribution of exchange, i.e., that of the lifetimes of the enzyme-bound metal–substrate complexes to the observed relaxation rates, should be determined on the basis of relaxation measurements as a function of temperature and frequency and not on the basis of line-width measurements; and (iv) sample conditions should be chosen such that the enzyme-bound complexes make as large a contribution as possible to observed relaxation rates, especially if the cation binds the substrate in free solution with an affinity comparable to that for the enzyme–substrate complex.

In this paper, ^{31}P spin relaxation measurements on the enzyme-bound substrate complexes of yeast 3-phosphoglycerate kinase in the presence of Mn(II) and Co(II) are presented. These experiments were aimed at determining the cation–^{31}P distances in different enzyme complexes and were performed by adapting the experimental strategy developed in the work on creatine kinase (Jarori et al., 1985) mentioned above. Metal–nucleotide interactions at the active site of 3-P-glycerate kinase have been probed in a number of investigations (Dunaway-Mariano & Cleland, 1980; Jaffe et al., 1982). X-ray diffraction studies of crystals of enzyme from horse

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; E·M·S, enzyme–metal–substrate; E·S, enzyme–substrate; EPR, electron paramagnetic resonance.

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muscle (Banks et al., 1979; Blake & Rice, 1981; Rice & Blake, 1984) and yeast (Bryant et al., 1974; Watson et al., 1982) soaked with Mg(II) or Mn(II) complexes of ADP and ATP have concluded that while the cation is directly coordinated to the two phosphate groups of ADP bound to the enzyme and the carboxylate group of an aspartyl residue on the enzyme, it is coordinated just to the γ-P of ATP and not to the other two phosphate groups. EPR measurements of superhyperfine interaction between ^{17}O regiospecifically labeled on ADP and Mn(II) in E·MnADP complexes (Moore & Reed, 1985) show general agreement with the X-ray crystallographic results for the ADP complexes. Similar EPR measurements with ^{17}O-labeled ATP are unable to provide unequivocal evidence regarding the Mn(II) chelate structure in the E·MnATP complexes. Ammonium sulfate crystallization was used for the X-ray work. The enzyme crystals, therefore, contained ammonium sulfate in excess of 2 M. Sulfate ion is known to measurably interfere with substrate binding at the active site of 3-P-glycerate kinase. In order to make a proper comparison between the results obtained in this work and the chelate structures of enzyme-bound metal–nucleotide complexes proposed by X-ray crystallography, the effect of sulfate ion on the ^{31}P NMR parameters of MgADP, MgATP, E·MgADP, and E·MgATP (some of these parameters are particularly sensitive to cation binding to the nucleotide) have been measured. These results were presented in the preceding paper (Ray & Nageswara Rao, 1988).

One factor that must be taken into account in the analysis of NMR measurements reported here is that ATP binds at two sites on the enzyme. ATP bound at one of these sites, which appears not to be the active site, has a much weaker affinity for Mg(II) compared to the other. The sensitivity of the line shape of the β-P (ATP) signal to Mg(II) binding permitted reasonable estimates of the various equilibrium constants relevant for the two ATP sites to be made. These results were also reported in the preceding paper (Ray & Nageswara Rao, 1988).

^{31}P spin relaxation measurements of the nucleotide complexes of creatine kinase in the presence of Mn(II) indicate that the lifetimes of the complexes are such that these relaxation rates are exchange-limited for cation–^{31}P distances up to about 6.5 Å. For distances shorter than this, Co(II) had to be used to obtain distance-dependent relaxation rates. In the case of 3-P-glycerate kinase, ^{31}P is also contained in the second substrate, viz., 3-P-glycerate, and this nucleus is expected to be at a distance farther than 6.5 Å from Mn(II) located near the nucleotide. Among the results presented below are relaxation measurements on the E·MnADP·3-P-glycerate complex that enabled the measurement of the Mn(II)–^{31}P (3-P-glycerate) distance in this complex. These results illustrate the methodological feature of exchange-limited relaxation rates for nuclei in close proximity and distance-dependent relaxation rates for nuclei located farther off in the same complex.

Experimental Procedures

Materials. Most of the materials and methods used in the work presented in this paper (e.g., enzyme preparation, assay, and NMR measurements) are identical with those in the preceeding paper (Ray & Nageswara Rao, 1988). Given below are the details of experimental conditions exclusively used for results in this paper. $MnCl_2$ solution (0.1 M) in 0.15 M NaCl was purchased from Sigma and $CoCl_2$ from Mallinckrodt.

NMR Measurements. ^{31}P NMR measurements at 121.5 MHz were made on an NT-300 wide-bore NMR spectrometer described briefly in the preceding paper (Ray & Nageswara Rao, 1988). Measurements at 81 and 190.2 MHz were made on NTC-200 and NTC-470 spectrometers at the Purdue University Biochemical Magnetic Resonance Laboratory. T_1 measurements were made by using a standard inversion–recovery sequence with a composite π pulse (Levitt, 1982) for inversion. The errors quoted for relaxation rates and activation energies are based on standard deviations given by computer fits and deviations between measurements made with independent samples.

Theoretical Details. Extensive reviews exist for the theory of nuclear spin relaxation in the presence of paramagnetic cations (Dwek, 1973; James, 1973; Mildvan & Gupta, 1978; Burton et al., 1979; Jardetzky & Roberts, 1981). A summary of this theory and the experimental strategy arising from it has been previously published (Jarori et al., 1985). The equations relevant for the analysis of data in this paper are given below. For a sample containing two exchanging complexes, one paramagnetic and the other diamagnetic, with fractional concentrations p and $(1 - p)$ and relevant nuclear relaxation rates $(T_{1M})^{-1}$ and $(T_{1D})^{-1}$, respectively, such that $(T_{1M})^{-1} \gg (T_{1D})^{-1}$, the observed relaxation rate is given by[2]

$$(T_{1,obsd})^{-1} = \frac{(1-p)}{T_{1D}} \frac{T_{1M} + \tau_M}{T_{1M} + (1-p)\tau_M} + \frac{p}{T_{1M} + (1-p)\tau_M} \quad (1)$$

where τ_M is the lifetime of the paramagnetic complex. If $p \ll 1$, eq 1 reduces to the commonly used form for the paramagnetic contribution $(T_{1P})^{-1}$ to $(T_{1,obsd})^{-1}$

$$(T_{1P})^{-1} = p/(T_{1M} + \tau_M) \quad (2)$$

T_{1M} is related to the cation–nucleus distance (neglecting the contribution of scalar hyperfine interaction) by

$$(T_{1M})^{-1} = (C/r)^6 f(\tau_C) \quad (3)$$

where

$$C = [(2/15)S(S+1)g^2\gamma_I^2\beta^2]^{1/6} \quad (4)$$

$$f(\tau_C) = 3\tau_{C1}/(1 + \omega_I^2\tau_{C1}^2) \quad (5)$$

for Mn(II) complexes (with $\omega_S\tau_{C2} \gg 1$), and

$$f(\tau_C) = 3\tau_{S1} + 7\tau_{S2}/(1 + \omega_S^2\tau_{S2}^2) \quad (6)$$

for Co(II) complexes with $(\tau_{Ci} = \tau_{Si})$, and

$$\tau_{Ci}^{-1} = \tau_R^{-1} + \tau_{Si}^{-1} \qquad i = 1, 2 \quad (7)$$

In eq 4–7, S, g, and ω_S are respectively the spin, the g-factor, and the Larmor frequency for the cation, γ_I and ω_I are respectively the gyromagnetic ratio and the resonance frequency of the relaxing nucleus, β is the Bohr magneton, τ_R is the isotropic rotational correlation time of the complex, and τ_{S1} and τ_{S2} are the electronic longitudinal and transverse relaxation times of the paramagnetic cation. These equations assume an isotropic g-factor and that the zero-field splitting is smaller than the Zeeman interaction of the cation, both of which are acceptable for complexes with Mn(II) but not for those with Co(II). For Mn(II) complexes, τ_{S1} is given by

$$(\tau_{S1})^{-1} = B[\tau_V/(1 + \omega_S^2\tau_V^2) + 4\tau_V/(1 + 4\omega_S^2\tau_V^2)] \quad (8)$$

[2] This expression represents the smaller of the two rates (λ) given by the roots of the equation

$$\begin{bmatrix} T_{1D}^{-1} + p/(1-p)\tau_M - \lambda & -\tau_M^{-1} \\ p/(p-1)\tau_M & T_{1M}^{-1} + \tau_M^{-1} - \lambda \end{bmatrix} = 0$$

with the approximation $T_{1D} \gg T_{1M}$.

Table I: Paramagnetic Effect $(pT_{1P})^{-1}$ of Mn(II) on ^{31}P Relaxation Rates and Corresponding Activation Energies (ΔE) for Various ADP and ATP Complexes Free in Solution and Bound to 3-P-glycerate Kinase[a]

complex (sample composition)	^{31}P NMR frequency (MHz)	α-P $(pT_{1P})^{-1}$ (s^{-1})	α-P ΔE (kcal/mol)	β-P $(pT_{1P})^{-1}$ (s^{-1})	β-P ΔE (kcal/mol)	γ-P/3-P $(pT_{1P})^{-1}$ (s^{-1})	γ-P/3-P ΔE (kcal/mol)
MnADP[b] (ADP, 5 mM; $MnCl_2$, 2.5–15 μM)	121.5	5400 ± 300		7200 ± 500			
MnATP[b] (ATP, 4 mM; $MnCl_2$, 2.5–15 μM)	121.5	5570 ± 200		7270 ± 200		6930 ± 200	
E·MnADP[c] (enzyme, 5.5 mM;	121.5	191 ± 20	6.4 ± 0.4	195 ± 20	7.8 ± 0.5		
ADP, 2.8 mM; $MnCl_2$, 5–50 μM)	190.2	197 ± 20		196 ± 20			
E·MnATP[c] (enzyme, 5.4 mM;	121.5	1060 ± 100	5.8 ± 0.4	1630 ● 100	6.0 ± 0.5	1290 ± 150	5.9 ± 0.4
ATP, 2.8 mM; $MnCl_2$, 4–20 μM)	190.2	1060 ± 100		1590 ± 100		1650 ± 200	
E·MnADP·3-P-glycerate[d] (enzyme,	81.0					68 ± 5	
6.2 mM; 3-P-glycerate, 2.5 mM;	121.5	170 ± 15		180 ± 20		48 ± 2	1.8 ± 0.2
ADP, 2.6 mM; $MnCl_2$, 50–90 μM)	190.2					20 ± 5	

[a] Samples for this work were in 200 mM K-HEPES, pH 8.2. The $(pT_{1P})^{-1}$ values given were measured at 5 °C. The ΔE values were obtained from Arrhenius plots (see Figures 1 and 2) of $(pT_{1P})^{-1}$ in the temperature range 5–20 °C. The errors were estimated on the basis of computer fit of the T_1 data and of the appropriate functions involved in obtaining final values. [b] Data for MnADP and MnATP were taken from Jarori et al. (1985). [c] Measurements were made with four to six values of p = [Mn(II)]/[nucleotide]. [d] Measurements were made with four to six values of p = [Mn(II)]/[3-P-glycerate]. $(pT_{1P})^{-1}$ for 3-P unchanged for 25% excess of 3-P-glycerate over ADP. The paramagnetic complexes are thus primarily quaternary.

where B is a constant related to the crystal field and τ_V is the correlation time for its fluctuation. For Co(II) complexes, however, eq 8 is unlikely to be valid, and even eq 6 may not be accurate.

The measurements presented below are made exclusively on enzyme-bound substrate complexes, which limit the exchange to two complexes, E·S and E·M·S, as implied in eq 1, and maximize the contribution of E·M·S to $(T_{1P})^{-1}$. Since structural information is not available from measurements in which $\tau_M \gg T_{1M}$, it is imperative to determine the contribution of τ_M to T_{1P} (see eq 1 and 2). This was done by making T_{1P} measurements as a function of temperature in the range 5–20 °C and at three ^{31}P frequencies (80, 121, and 190 MHz). Note that the activation energies of T_{1M} are usually 1–3 kacl/mol while those for τ_M are 5–20 kcal/mol. Furthermore, T_{1M} depends on frequency, and τ_M does not.

Results and Analysis

Mn(II)–Nucleotide Complexes. The values of $(pT_{1P})^{-1}$ obtained for the ^{31}P nuclei in the phosphate groups of E·MnADP and E·MnATP at 5 °C are given in Table I along with previously published data for MnADP and MnATP (Jarori et al., 1985). The data given are based on measurements made at 121.5 and 190.2 MHz. For the enzyme complexes, on the basis of estimated (Ray & Nageswara Rao, 1988) and previously published (Scopes, 1978) dissociation constants, the fractional concentration of the paramagnetic complexes free in solution ([M·S]/[E·M·S]) never exceeds ~3%. It has been recognized for some time that there are two ATP-binding sites on 3-P-glycerate kinase (Larsson-Raznikiewicz & Schierbeck, 1977; Scopes, 1978; Nageswara Rao et al., 1978). One of these sites has very little affinity for MgATP, and in the absence of the divalent cation, the ^{31}P chemical shifts of bound nucleotides are independent of which of the two binding sites the nucleotide occupies. The binding constants determined suggest that nucleotides bound at the two sites will always be in fast exchange (Ray & Nageswara Rao, 1988). Thus, the values of $T_{1,obsd}$ measured represent the total relaxation of all nucleotide bound to the enzyme, while the enhancement of relaxation by paramagnetic cations occurs only at the tighter binding site. Therefore, for all complexes, the appropriate fractional concentration, p = [E·M·S]/[E·S], is the ratio of paramagnetic cation to total nucleotide.

As was the case with creatine kinase (Jarori et al., 1985), the ^{31}P relaxation rates of α-P and β-P of E·MnADP and those

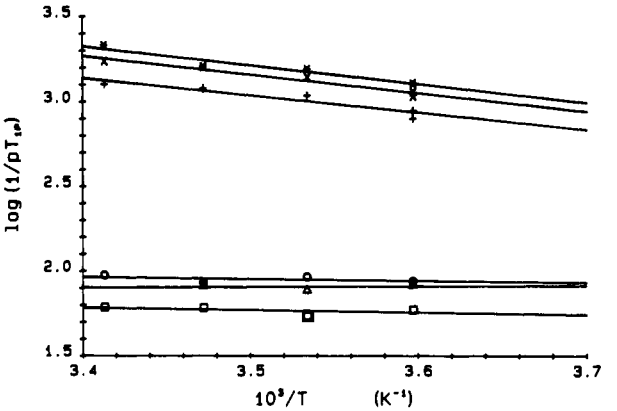

FIGURE 1: log $(pT_{1P})^{-1}$ vs $10^3/T$ for α-P, β-P, and γ-P of E·MnATP (+, *, ×) and E·CoATP (□, △, ○). Typical sample conditions and activation energies (ΔE) obtained are given in Tables I and II.

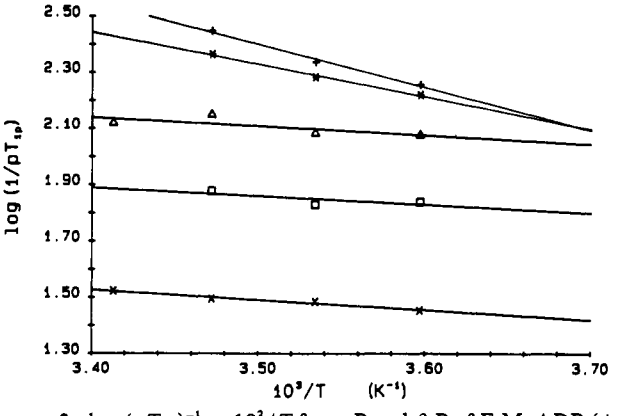

FIGURE 2: log $(pT_{1P})^{-1}$ vs $10^3/T$ for α-P and β-P of E·MnADP (+, *) and E·CoADP (□, △) and for 3-P of 3-P-glycerate in E·MnADP·3-P-glycerate (×). Typical sample conditions and activation energies (ΔE) obtained are given in Tables I and II.

of α-P, β-P, and γ-P of E·MnATP are nearly equal and are unchanged within experimental error between 121.5 and 190.2 MHz, indicating that these rates are exchange-limited. Further evidence for exchange limitation of ^{31}P relaxation times in these complexes is obtained from their temperature dependence in the range 5–20 °C (Figures 1 and 2). Arrhenius plots of $(pT_{1P})^{-1}$ yield activation energies, ΔE, in the range 5–8 kcal/mol for the ^{31}P nuclei in E·MnADP and E·MnATP (see Table I). These values are in the range appro-

Table II: Paramagnetic Effect $(pT_{1P})^{-1}$ of Co(II) on ^{31}P Relaxation Rates and Corresponding Activation Energies ΔE for Various ADP and ATP Complexes Free in Solution and Bound to 3-P-glycerate Kinase[a]

complex (sample composition)	^{31}P NMR frequency (MHz)	α-P $(pT_{1P})^{-1}$ (s^{-1})	α-P ΔE (kcal/mol)	β-P $(pT_{1P})^{-1}$ (s^{-1})	β-P ΔE (kcal/mol)	γ-P $(pT_{1P})^{-1}$ (s^{-1})	γ-P ΔE (kcal/mol)
CoADP[b]	121.5	280 ± 20		560 ± 50			
CoATP[b] (ATP, 2.7 mM; $CoCl_2$, 15–25 μM)	121.5	120 ± 20		150 ± 20		210 ± 20	
E·CoADP (enzyme, 4.9 mM; ADP,	81.0	90 ± 10		153 ± 20			
2.7 mM; $CoCl_2$, 40–380 μM)	121.5	203 ± 20	2.9 ± 0.2	243 ± 20	2.3 ± 0.2		
	190.2	104 ± 10		148 ± 10			
E·CoATP (enzyme, 5.0 mM; ATP,	81.0	100 ± 10		208 ± 20		171 ± 10	
3.0 mM; $CoCl_2$, 50–300 μM)	121.5	102 ± 10	1.4 ± 0.1	163 ± 15	1.4 ± 0.1	185 ± 15	1.3 ± 0.1
	190.2	60 ± 10		70 ± 10		128 ± 10	

[a] Samples for this work were in 200 mM K-HEPES, pH 8.2. The $(pT_{1P})^{-1}$ values given were measured at 5 °C with at least four different values of p = [Co(II)]/[nucleotide]. Measurements were made at 81, 121.5, and 190.2 MHz. The ΔE values were obtained at 121.5 MHz from Arrhenius plots (see Figures 1 and 2) of $(pT_{1P})^{-1}$ in the temperature range 5–20 °C. The errors were estimated on the basis of computer fits of the T_1 data and of the appropriate functions involved in obtaining the final values. [b] CoADP and CoATP data were taken from Jarori et al. (1985).

priate for τ_M and are much larger than would be expected for τ_R or τ_V. Thus, the ^{31}P relaxation data in the Mn(II)–nucleotide complexes bound to 3-P-glycerate kinase are determined primarily by the lifetimes of these complexes and not by the distances between the cation and the ^{31}P nuclei.

Co(II)–Nucleotide Complexes. Values of $(pT_{1P})^{-1}$ obtained for ^{31}P nuclei in the nucleotide phosphate groups of E·CoATP and E·CoADP at 5 °C are given in Table II along with previously published data for CoADP and CoATP (Jarori et al., 1985). Measurements were made at three frequencies, viz., 81, 121.5, and 190.2 MHz. Temperature dependence of $(pT_{1P})^{-1}$ at 121.5 MHz was measured in the range 5–20 °C. Arrhenius plots for E·CoADP and E·CoATP are included in Figures 1 and 2, respectively. The values of ΔE are given in parentheses in Table II. For E·CoADP and E·CoATP, the ΔE values are in the range 1–3 kcal/mol and are significantly lower than for the Mn(II)–nucleotide complexes. Furthermore, the $(pT_{1P})^{-1}$ values for these complexes show a small but reproducible frequency dependence. The relaxation rates in these two Co(II) complexes are, therefore, primarily determined by T_{1M}, and the contribution of τ_M is much smaller.[3]

In order to calculate the cation–^{31}P distances from the relaxation rates in Table II, values of $f(\tau_C)$ for the various complexes are needed (see eq 3). For the Co(II) complexes, $f(\tau_C)$ is determined exclusively by the electron relaxation times (eq 6), which are in the neighborhood of 10^{-12} s. Since $f(\tau_C)$ does not depend on τ_R, its value will not depend on the size of the protein and for similar complexes may not vary much from protein to protein. There are a number of theoretical problems, however, in making accurate estimates of $f(\tau_C)$ (Benetis et al., 1983; Jarori et al., 1985). Nevertheless, the range 10^{-12} s $< f(\tau_C) \leq 5 \times 10^{-12}$ s was used to analyze the 121.5-MHz relaxation data for Co(II) complexes of creatine kinase (Jarori et al., 1985) on the basis of the frequency dependence exhibited by the relaxation rates as well as previously published data for various enzyme complexes with Co(II) (Mildvan et al., 1980; Villafranca, 1984). The relaxation data in Table II also show frequency sensitivity in the range 80–190 MHz for ^{31}P, i.e., $\omega_S = 1.7$–4.2×10^{12} rad s^{-1} for Co(II) (with $g = 4.33$). Thus, the same range as above appears appropriate. The distances are therefore calculated by using the range 10^{-12} s $< f(\tau_C) \leq 5 \times 10^{-12}$ s and $C = 675$ Å $s^{-1/3}$ in eq 3. The range of values chosen for $f(\tau_C)$ is expected to absorb any other corrections (Sternlicht, 1965; Rubenstein et al., 1971) such as that due to the anisotropic g-tensor of Co(II) (Vasavada & Nageswara Rao, 1988). The distances signify direct coordination of Co(II) with all the phosphate groups in both

[3] The value of T_{1M} for both the ^{31}P nuclei in E·CoADP is ~5 ms at 121.5 MHz (see Table II). The corresponding τ_M value is thus likely to be <1 ms. On the other hand, the τ_M values for the exchange-limited relaxation rates in E·MnADP are about 5 ms (see Table I). Similarly, the τ_M values estimated for MnADP bound to creatine kinase were at least 5-fold larger than those for the bound CoADP (Jarori et al., 1985).

Table III: Distances of ^{31}P Nuclei from the Cation [Mn(II) or Co(II)] in Various Complexes of 3-P-glycerate Kinase and in CoADP and CoATP

complexes	cation–^{31}P distance (Å) α-P	β-P	γ-P	3-P
CoADP[a]	2.6–3.5	2.4–3.1		
CoATP[a]	3.0–4.0	2.9–3.8	2.7–3.6	
E·CoADP[b]	2.8–3.6	2.7–3.5		
E·CoATP[b]	3.1–4.1	2.9–3.8	2.8–3.7	
E·MnADP·3-P-glycerate[c]				11.1 ± 0.3

[a] Data for CoADP and CoATP taken from Jarori et al. (1985). [b] Calculations are based on $(pT_{1P})^{-1}$ data at 121.5 MHz using eq 3 with $C = 675$ Å $s^{-1/3}$ and 10^{-12} s $\leq f(\tau_C) \leq 5 \times 10^{-12}$ s. Distances in Co(II) complexes are given as a range corresponding to the range chosen for $f(\tau C)$. Errors arising from $(pT_{1P})^{-1}$ are ~2–4%. Most of the uncertainty in the distances is due to the estimation of $f(\tau_C)$. [c] Distance was calculated on the basis of the frequency dependence of the relaxation times, pT_{1P}, in the range 81–190.2 MHz (see Table I) and $C = 601$ Å $s^{-1/3}$ (see text).

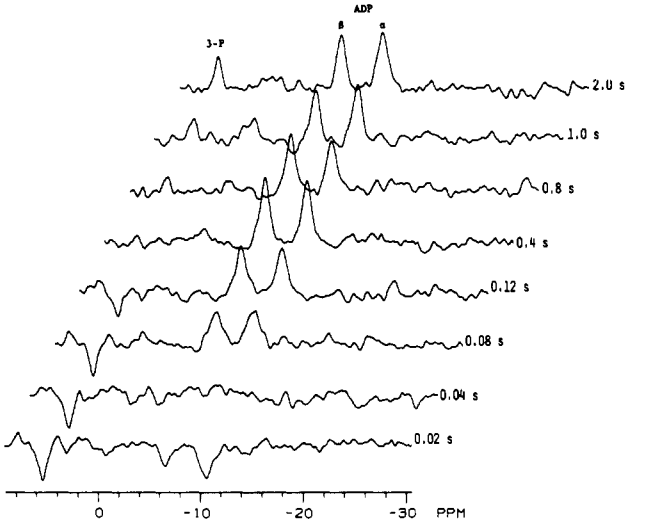

FIGURE 3: Typical T_{1P} measurements at 121.5 MHz for ^{31}P nuclei in ADP and 3-P-glycerate bound to 3-P-glycerate kinase in the presence of Mn(II) ($p = 0.114$) at $T = 5$ °C. A standard inversion–recovery sequence was used with a composite π pulse. NMR parameters: $\pi/2$ pulse width, 20 μs; sweep width, ±2400 Hz; data size, 2048; line broadening, 60 Hz; number of scans, 224; recycle delay, 2.4 s. Computer fit gives T_{1P} values of 0.05, 0.05 and 0.69 s (standard deviation ~0.01 s) for α-P, β-P, and 3-P, respectively.

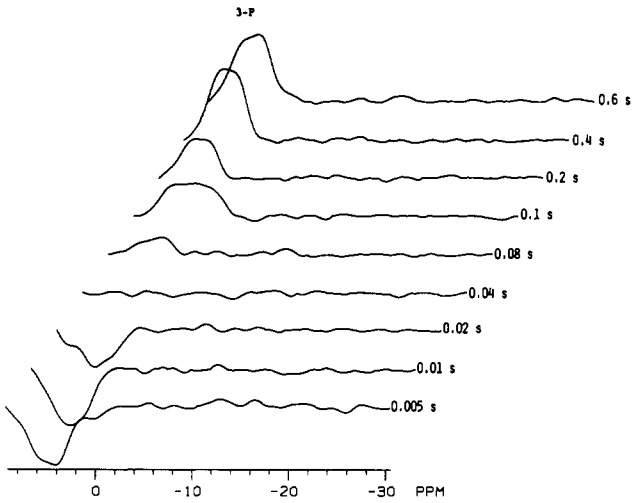

FIGURE 4: T_{1P} measurement at 121.5 MHz and high [Mn(II)] for ^{31}P nuclei in ADP and 3-P-glycerate bound to 3-P-glycerate kinase ([Mn]:[ADP] = 0.39, T = 5 °C). NMR parameters: line broadening, 250 Hz; number of scans, 512; recycle delay, 0.8 s; all other NMR parameters are as given for Figure 3. Computer fit gives 0.081 s as T_{1P} for 3-P of 3-P-glycerate. Signals from α-P and β-P of ADP are too broad to be observed.

ADP and ATP. (See Table III.)

E·MnADP·3-P-glycerate Complex. Values of $(pT_{1P})^{-1}$ obtained at 5 °C for the ^{31}P nuclei in this complex are given in Table I. A typical inversion–recovery stack plot used for the T_1 measurements is shown in Figure 3 for a sample in which p = [Mn(II)]/[ADP] = 0.114 and [ADP] = [3-P-glycerate]. It is evident that the ^{31}P signal of 3-P-glycerate relaxes much more slowly than those of the α-P and β-P of ADP in the complex. This disparity in the relaxation is strikingly illustrated in the stack plot in Figure 4, which is obtained at a concentration of Mn(II) such that p = 0.39. In the spectrum of Figure 4, the signals of α-P and β-P of ADP are too broad to be observed, whereas that of 3-P-glycerate is readily measurable. $(pT_{1P})^{-1}$ values for the three ^{31}P nuclei are given in Table I. The exchange-limited values of $(pT_{1P})^{-1}$ for α-P and β-P of E·MnADP show little change due to addition of 3-P-glycerate. Temperature dependence of $(pT_{1P})^{-1}$ for the 3-P-glycerate signal at 121.5 MHz shows a ΔE of 1.8 kcal/mol. Measurements of $(pT_{1P})^{-1}$ at 80 and 190 MHz clearly displayed a frequency dependence (see Table I). Thus, this relaxation rate depends on the cation–^{31}P distance and is not exchange-limited. The ^{31}P relaxation rates in E·MnADP·3-P-glycerate provide a vivid example of exchange limitation and distance dependence of paramagnetic relaxation being simultaneously manifest in the same complex. The two ^{31}P nuclei in ADP possess exchange-limited relaxation rates because of the direct coordination of the α-P and β-P with Mn(II), whereas the relaxation rate of the ^{31}P in 3-P-glycerate is clearly dependent on the distance because it is located at a much larger distance from the cation. Note, however, the presence of MnADP in the complex is required for measuring the distance of Mn(II) to the ^{31}P in 3-P-glycerate because the cation is chelated to ADP on the enzyme.

The frequency dependence of $(pT_{1P})^{-1}$ for the ^{31}P of 3-P-glycerate complex may be used to determine $f(\tau_C)$ and calculate the cation–^{31}P distance. Prior to this calculation, the value of τ_M appropriate for this ^{31}P nucleus should be assessed. It is clear that the exchange-limited values of $(pT_{1P})^{-1}$ for α-P and β-P of ADP in this complex imply a τ_M of about 5.7 ms for these ^{31}P nuclei. Is this value also appropriate for the τ_M of the ^{31}P of 3-P-glycerate? The ΔE value of 1.8 kcal/mol for $(pT_{1P})^{-1}$ is in the middle of the range of 1–3 kcal/mol

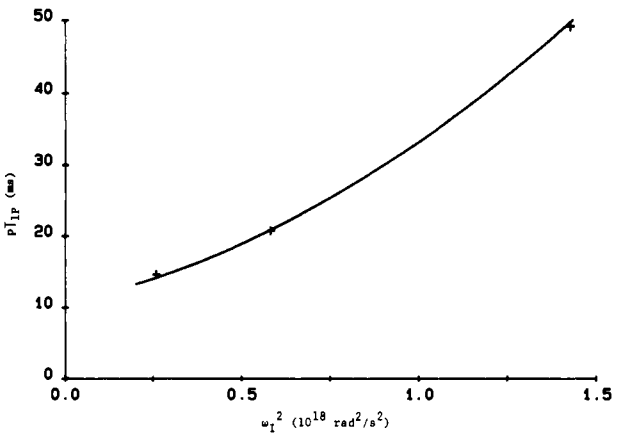

FIGURE 5: pT_{1P} (ms) vs ω_I^2 (10^{18} rad^2 s^{-2}) for 3-P of 3-P-glycerate in E·MnADP·3-P-glycerate. Experimental data points shown are for 81, 121.5, and 190 MHz. Theoretical curve is for $B = 5.9 \times 10^{20}$ s^{-2}, $\tau_V = 2.8 \times 10^{-13}$ s, C = 601 Å s$^{-1/3}$, τ_M = 0, and r = 11.1 Å.

normally expected for τ_R or τ_V, suggesting that τ_M is not a major part of the relaxation time of 20.8 ms measured for this nucleus at 121.5 MHz. This evidence is not, however, adequate to make a quantitative estimate of τ_M. It may be noted that the value of τ_M appropriate for the ^{31}P of 3-P-glycerate is determined by the exchanges between E·MnADP·3-P-glycerate and those enzyme-bound diamagnetic complexes in which 3-P-glycerate is present. Such a τ_M value may, in general, be different from that for α-P and β-P of ADP in this complex. In particular, the lifetimes of dissociation and reassociation of 3-P-glycerate in this complex enter the τ_M value of this substrate, whereas these steps make negligible contributions for the τ_M values of ADP [note that Mn(II) is predominantly in the form bound to ADP]. If these lifetimes are significantly shorter than the 5.7 ms obtained for ADP, the τ_M value for 3-P-glycerate will be shorter. In any event, the upper limit for this τ_M is 5.7 ms.

The lifetime of 3-P-glycerate in the complex E·MgADP·3-P-glycerate may be estimated from the ^{31}P chemical shift data. At pH 8.2, the chemical shift of 3-P-glycerate in the enzyme-bound quaternary complex differs from that in free solution by ~0.5 ppm. In the presence of a sufficient excess of 3-P-glycerate the ^{31}P NMR spectrum failed to reveal separate signals for free and bound 3-P-glycerate, indicating that these signals are in fast exchange, i.e., reciprocal lifetime larger than 382 s^{-1}. This places an upper limit of 2.6 ms on the lifetime. This estimate may be reasonable for the Mn(II) complex as well because the dissociation constant for 3-P-glycerate appears to be insensitive to the cation used (Scopes, 1978).

In order to determine $f(\tau_C)$ and τ_M consistent with experimental data, the following procedure is used. The pT_{1P} values for 3-P-glycerate of E·MnADP·3-P-glycerate are plotted against ω_I^2 and are shown in Figure 5. It may be seen from eq 5 that if τ_{C1} is independent of the external magnetic field, a plot of pT_{1P} vs ω_I^2 will be linear with a slope given by $^1/_3(r/C)^6\tau_{C1}$ and a y intercept equal to $[^1/_3(r/C)^6\tau_{C1}^{-1} + \tau_M]$. Therefore, τ_{C1} may be calculated from the slope and the intercept if τ_M is known. However, if τ_{S1} contributes to τ_{C1} and the condition $\omega_S\tau_V \ll 1$ does not hold (see eq 8), the plot becomes nonlinear because of the composite field dependence of ω_I and τ_S. The data shown in Figure 5 may be approximated by a linear fit, although there is a noticeable curvature in the low ω_I region. Therefore, the data were analyzed both ways, (i) by considering τ_{C1} to be field independent (linear plot) and neglecting the contribution of τ_M to the y intercept, and (ii) by considering a field-dependent τ_{C1} as given by eq 7 and

8 and making a numerical fit of the data with different values of B, τ_V, r, and different values τ_M from 0 to 3.0 ms. The linear plot yields $\tau_{C1} = 2.4 \pm 0.1$ ns and $r = 10.8 \pm 0.5$ Å [$C = 601$ Å $s^{-1/3}$ was used for Mn(II) complexes]. The nonlinear plot fits best with the parameters in the range $B = (5.4 \pm 0.5) \times 10^{20}$ s^{-2}, $\tau_V = (3.2 \pm 0.4) \times 10^{-13}$ s, for different values of $\tau_M < 2.5$ ms, and $r = 11.1 \pm 0.3$ Å. The calculated T_{1M} vs ω_I^2 plot is shown as the solid curve in Figure 5. The distance, r, is not much affected either by the value chosen for τ_M or by whether the field dependence of τ_{C1} is considered or not. This is a fortunate consequence of the sixth power in the exponent of r in eq 3.

The correlation time for ^{31}P relaxation in the presence of Mn(II) appears to be in the range 1–5 ns for these complexes. This value is too short to be governed by τ_R, which is expected to be an order of magnitude larger for a globular protein of molecular weight 47 000. It is, therefore, likely to be primarily determined by τ_{S1}, the electronic longitudinal relaxation time. A substantial contribution of τ_{S1} to τ_{C1} for E·MnADP and E·MnADP·3-P-glycerate complexes was indicated from previously published data on enhancement of proton relaxation rates of solvent water (Chapman et al., 1977).[4]

Discussion

The structure-dependent ^{31}P relaxation rates of CoADP and CoATP bound to 3-P-glycerate kinase show that the cation is directly coordinated to all the phosphate groups of the nucleotide in both of these complexes. The direct coordination of the cation to α-P and β-P of ADP bound to 3-P-glycerate kinase is in agreement with the results of X-ray crystallography (Bryant et al., 1974; Watson et al., 1982) and Mn(II) EPR (Moore & Reed, 1985) measurements. However, for ATP bound to this enzyme, the conclusion of direct coordination of the cation to all three phosphate groups does not agree with the X-ray results that place the Mg(II) ion coordinated just to γ-P (ATP) and away from γ-P and β-P (Bryant et al., 1974; Watson et al., 1982). The evidence from the ^{31}P relaxation measurements is unequivocal with respect to direct coordination, even though the Co(II)–^{31}P distances could only be given as a range. The observed relaxation rates are similar for β-P and γ-P, and these rates are larger than those for α-P. However, such a difference does not necessarily imply closer proximity of the cation to γ-P and β-P in preference to α-P in view of the anisotropy of the g-tensor for Co(II).[3] In any event, because of the dependence of the relaxation rates on r^{-6}, the difference between these distances is too small to alter the conclusion of direct coordination between the cation and all three phosphate groups. On the other hand, the X-ray measurements were made on crystals grown in ammonium sulfate (Bryant et al., 1974; Watson et al., 1982). On the basis of the results of the preceding paper (Ray & Nageswara Rao, 1988), it is most likely that the results of X-ray measurements of the chelate structure of E·MgATP were perturbed by the presence of over 2 M sulfate ion in the crystals. The fact that such a perturbation occurs for E·MgATP and not for E·MgADP appears to be related to the fact that the cation is known to bind to E·ADP with greater affinity than it does to E·ATP (Scopes, 1978). This may also account for the agreement between X-ray (Bryant et al., 1974; Watson et al., 1982) and Mn(II) EPR (Moore & Reed, 1985) measurements on the MnADP complex with the enzyme.

The pattern of direct coordination of the cation to all the phosphate groups of ADP as well as ATP bound to 3-P-glycerate kinase is in agreement with similar results obtained for the complexes of creatine kinase (Leyh et al., 1985; Jarori et al., 1985) and arginine kinase.[5] As has been discussed in some detail (Jarori et al., 1985; Nageswara Rao, 1987), distances appropriate for second-coordination sphere or longer may be obtained if the contribution of τ_M to $(pT_{1P})^{-1}$ is incorrectly assessed for relaxation rate due to directly coordinated Mn(II). The importance of making a reasonable estimate of τ_M can hardly be overemphasized if the structure data obtained by relaxation measurements in the presence of paramagnetic cations are to carry any reliability at all. Often, it may be necessary to determine the role of τ_M by more than one method, such as temperature and frequency dependency, and by using more than one paramagnetic cation if possible. The protocol of making measurements with substrates exclusively in the enzyme-bound form significantly enhances the accuracy and reliability of the relevant paramagnetic relaxation rates and of the analysis of variations in them.

The experiments on the quaternary complex E·MnADP·3-P-glycerate displayed a feature that is expected to occur when this methodology is used on complexes in which the nuclei are located at widely differing distances from the cation, viz., the simultaneous occurrence of exchange limitation for some relaxation rates and structure dependence for the others. In this complex, the ^{31}P relaxation rates of ADP were exchange-limited, while those of 3-P-glycerate were not. A closer examination of the measured rates indicated that the value of τ_M for 3-P-glycerate appears to be primarily governed by its dissociation from the enzyme, whereas that for ADP is determined by the relatively slower steps involving the dissociation of either MnADP from the enzyme or Mn(II) from E·ADP. Nevertheless, MnADP is required to make distance measurements to the ^{31}P of 3-P-glycerate as it holds the cation in the complex. Thus, these results shed some light on the factors to be recognized in using this method for measuring distances from the cation to nuclei located at distances larger than ~4–5 Å either on the substrate to which it is chelated or present in substrates (or inhibitors) at the active site which do not directly bind the cation to a significant extent (either off or on the enzyme). When the distances are longer than about 7 Å, relaxation rates in the presence of Mn(II) are likely to overcome the lifetimes and become structure dependent as in the case of 3-P-glycerate in E·MnADP·3-P-glycerate. Such distances can be determined with greater precision than those for the Co(II) complexes (at closer distances) because Mn(II) is not beset with the theoretical problems associated with Co(II) regarding such factors as $f(\tau_C)$ and g-tensor anisotropy. If it were possible to measure distant-dependent relaxation rates with both the cations, the uncertainty in the analysis of the data on Co(II) complexes may be circumvented by using the results on Mn(II) complexes to "calibrate" $f(\tau_C)$ for the Co(II) complexes. This approach is likely to be possible for

[4] These measurements were made at fields corresponding to the range 20–60 MHz for proton resonance frequencies, whereas the data in the present work are obtained in the 200–470-MHz range. If there is a single τ_{S1} for the cation with a field dependence given by eq 8, the values of τ_{S1} (~5 ns) taken from the results of Chapman et al. (1977) and extrapolated to the higher fields in the present work would easily exceed τ_R of 3-P-glycerate kinase expected to be in the range 50 ± 30 ns. In such a case, the effective correlation time for the present data should be τ_R (see eq 7). The fact that the analysis presented shows τ_{S1} to be dominant even at the higher fields suggests a more complex behavior of τ_{S1} as a function of frequency (including the possibility of multiple values for τ_{S1}) than is implied by the simple relation given by eq 8. We wish to thank Dr. G. K. Jarori for bringing this point to our attention. (Added in proof.)

[5] B. D. Ray, G. K. Jarori, and B. D. Nageswara Rao, unpublished experiments.

cation–nucleus distances in the range 5–7 Å[6] and could not, therefore, be used for the case of the ^{31}P relaxation rate of 3-P in E·MnADP·3-P-glycerate, where the cation–nucleus distance is about 11 Å. At such a distance the effect of Co(II) on the relaxation rate is too feeble to be detectable.

The distance of 11.1 ± 0.3 Å between Mn(II) and the ^{31}P of 3-P-glycerate in E·MnADP·3-P-glycerate suggests, on the basis of a Dreiding molecular model, an open conformation for the active site in which there is a gap between the oxygens of the γ-P (ATP) and of the carboxyl group of 3-P-glycerate that is to receive the transferable phosphoryl group. In the framework of the hinge-bending hypothesis (Bryant et al., 1974), this suggests that the hinge may not be closed in this complex. The formation of the reaction complex, i.e., the presence of MnATP rather than MnADP, may be required for the hinge bending to take place. A similar conclusion was reached from the Mn(II) EPR results (Moore & Reed, 1985). This conclusion can be tested if the Mn(II)–^{31}P (3-P-glycerate) distance can be measured in the reaction complex E·MnATP·3-P-glycerate. Unfortunately, the method of paramagnetic effects on nuclear spin relaxation is not yet sufficiently well honed to properly analyze relaxation data of enzyme bound reaction complexes. This will require taking into account the additional exchange effects that arise from the interconversion of enzyme-bound reactants and products. Attempts to develop such a procedure are in progress.

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