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Structure of Metal–Nucleotide Complexes Bound to Creatine Kinase: ^{31}P NMR Measurements Using Mn(II) and Co(II)†

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ABSTRACT: The structures of metal–nucleotide complexes bound to rabbit muscle creatine kinase have been studied by making measurements of paramagnetic effects of two dissimilar activating paramagnetic cations, Mn(II) and Co(II), on the spin-relaxation rates of the ^{31}P nuclei of ATP and ADP in these complexes. The experiments were performed on enzyme-bound complexes, thereby limiting the contributions to the observed relaxation rate to two exchanging complexes (with and without the cation). Measurements were made as a function of temperature in the range 5–35 °C and at three ^{31}P NMR frequencies, 81, 121.5, and 190.2 MHz, in order to determine the effect of exchange on the observed relaxation rates. The relaxation rates in E·MnADP and E·MnATP are independent of frequency, and their temperature variation yields activation energies (ΔE) in the range 5–8 kcal/mol; in the transition-state analogue complex E·MnADP·NO_3^-·Cre (Cre is creatine), ΔE is increased to 17.3 kcal/mol. These results demonstrate that the relaxation rates in the Mn(II) complexes are exchange limited and are incapable of providing structural data. It is shown further that use of line-width measurements to estimate the lifetime of the paramagnetic complex leads to incorrect results. The relaxation rates in 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, whereas those in the E·CoADP·NO_3^-·Cre complex have $\Delta E \sim 18$ kcal/mol and are significantly contributed by exchange. Difficulties involved in estimating the electron relaxation times in E·CoADP and E·CoATP restrict the calculation of Co(II)–^{31}P distances in these complexes to lower and upper limits. These distances were all in the range 2.4–4.3 Å, similar to those for free complexes and appropriate for direct coordination between Co(II) and the phosphate groups. This conclusion is in agreement with that reached by using Mn(II) EPR on this enzyme [Leyh, T. S., Goodhart, P. J., Nguyen, A. C., Kenyon, G. L., & Reed, G. H. (1985) *Biochemistry 24*, 308–316] and is in contrast with the conclusion of second-hydration-sphere coordination for Mn(II)–nucleotide complexes of pyruvate kinase [Sloan, D. L., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2412–2420] reached by using ^{31}P relaxation measurements.

The role of obligatory divalent cations in enzymatic reactions utilizing ATP[1] has been a subject of continued interest. Since there are a variety of enzymes for which nucleotides are substrates, information on the structure of enzyme-bound nucleotide complexes under catalytic or exchange-inert conditions is of considerable value, not only because of its mechanistic implications but also because it affords a comparison of these structural features for different enzymes to gain insight into catalytic action. Several methods are being used for probing metal–nucleotide complexes at the active sites of enzymes in solution: (i) ^{17}O superhyperfine structure effects of selectively labeled ligands on Mn(II) EPR spectra (Reed & Leyh, 1980; Leyh et al., 1985); (ii) ^{17}O and ^{18}O isotope effects on ^{31}P NMR (Cohn, 1982; Tsai & Bruzik, 1983); (iii)

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

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use of exchange-inert $Cr^{III}(NH_3)_4$ or $Co^{III}(NH_3)_4$ complexes of nucleotides (Cleland & Mildvan, 1979; Dunaway-Mariano & Cleland, 1980); (iv) metal ion effects on the stereoselectivity of isomers of phosphorothioate analogues of nucleotides (Jaffe & Cohn, 1979; Tsai, 1984); (v) nuclear spin-relaxation measurements on substrate nuclei in the presence of substituent paramagnetic cations (Mildvan & Gupta, 1978; Villafranca, 1984). While methods i–iv are helpful in identifying the ligand nucleus attached to the cation, the last method is one of the few available for the determination of distances between the cation and the substrate nuclei. There are, however, a number of pitfalls in the determination of reliable data on the paramagnetic contribution to the relaxation of the substrate nucleus in the enzyme–metal–substrate (E·M·S) complex, T_{1M}^{-1}, and in the procedure for deriving structure from T_{1M}^{-1} (Mildvan & Gupta, 1978; Burton et al., 1979; Mildvan et al., 1980).

Paramagnetic probes have been employed extensively in active site structure studies on a number of enzymes with substrate concentrations far in excess of the enzymes in all cases [for reviews, see Mildvan & Gupta (1978), Cohn & Nageswara Rao (1980), Mildvan et al. (1979), and Villafranca (1984)]. The choice of such experimental conditions implies the following: (i) the relaxation rate of the enzyme complex is measured as a difference of two relatively large rates (with and without the enzyme). (ii) The individual relaxation rates of four complexes, E·S, E·M·S, M·S, and S, and the exchange rates between them contribute to the observed rate. (iii) T_{1M}^{-1} is deduced by an extrapolation procedure that depends critically on the accuracy of the measurements and the validity of fast exchange; and such a validation is not unambiguously obtained with the sample conditions chosen. It is therefore difficult to ensure that the T_{1M}^{-1} deduced for E·M·S is reliable. Evidently, it is preferable to make measurements on enzyme-bound substrates, which limit contributions to the observed rate, primarily, to relaxation rates of two exchanging complexes (E·S and E·M·S). Since such measurements have never been made, neither were the methodological features of using paramagnetic probes well scrutinized, nor was the reliability of the conclusions obtained clearly delineated.

In this paper active site structure studies of metal–nucleotide complexes of rabbit muscle creatine kinase using ^{31}P relaxation measurements in the presence of Mn(II) and Co(II) are reported. The enzyme catalyzes the reaction

$$\text{ATP} + \text{creatine} \xrightleftharpoons{\text{Mg(II)}} \text{ADP} + \text{phosphocreatine} \quad (1)$$

with Mg(II) as the obligatory divalent cation (Watts, 1973; Kenyon & Reed, 1983). There is no evidence for a direct interaction of the cation with functional groups on the enzyme (Reed & Leyh, 1980; Dunaway-Mariano & Cleland, 1980). The feasibility of observing and analyzing ^{31}P NMR signals of enzyme-bound substrate complexes of creatine kinase was established in earlier studies with Mg(II) as the cation (Nageswara Rao, 1979, 1984; Vasavada et al., 1980; Nageswara Rao & Cohn, 1981). Substituent paramagnetic cations Mn(II) or Co(II) activate the enzyme. The choice of two dissimilar paramagnetic cations along with the restriction of the measurements to enzyme-bound complexes allows scrutiny of some of the methodological questions mentioned above.

Measurements of the effect of Mn(II) on the relaxation of protons of creatine in a transition-state analogue complex of creatine kinase (E·MnADP·$HCOO^-$·Cre) were previously used (McLaughlin et al., 1976) to suggest that Mn(II) is chelated to α-P and β-P of ATP. However, recent work of Reed et al. (1978) making use of infrared vibrational modes of anions used in the transition-state analogue complex and of Reed & Leyh (1980) and Leyh et al. (1985) making use of superhyperfine coupling between Mn(II) and ^{17}O (substituted on the phosphate groups) indicates direct coordination of Mn(II) with all three phosphate groups of ATP on the enzyme. On the other hand, structure determination of metal–nucleotide complexes at the active sites of rabbit muscle pyruvate kinase (Sloan & Mildvan, 1976; Gupta et al., 1976; Gupta & Benovic, 1978) using Mn(II) and Cr(III) as paramagnetic probes concludes upon a second-hydration-sphere coordination between Mn(II) and ATP. The measurements reported in this paper provide ^{31}P NMR evidence relevant to the examination of these conclusions, which are in disagreement.

Experimental Procedures

Materials. ADP, ATP, creatine, Hepes, and 0.1 M $MnCl_2$ solution in 0.15 M NaCl and rabbit muscle lactate dehydrogenase containing pyruvate kinase were purchased from Sigma Chemical Co. $CoCl_2$ was obtained from Mallinckrodt Inc. All other chemicals used were of analytical reagent grade. ADP, ATP, and buffer solutions were passed through a Chelex-100 column before use in the NMR experiments.

Enzyme Preparation. Creatine kinase was prepared from the skeletal muscles of a rabbit, by method B of Kuby et al. (1954). The purified enzyme had a specific activity of ~50 IU in a coupled assay with lactate dehydrogenase and pyruvate kinase at room temperature (Tanzer & Gilvarg, 1959). Appreciable levels of ATPase activity (Sasa & Noda, 1964) and an apparent hydrolysis of ADP were previously noticed at high creatine kinase concentrations (Nageswara Rao & Cohn, 1981). The enzyme was, therefore, subjected to gel filtration chromatography on a Sephadex G-75 column (45 cm × 2.5 cm). It was then precipitated by the addition of $(NH_4)_2SO_4$ to 90% saturation, dissolved in 20 mM K-Hepes buffer (pH 8.0), and dialyzed extensively against the same buffer containing preequilibrated Chelex-100. The specific activity of the enzyme at this stage was ~70 IU. Incubation of 3 mM ADP (or ATP) with 5 mM enzyme (sites) for ~8 h at 30 °C showed no hydrolysis with Co(II); with Mn(II), however, while ADP did not break down, ~30% of ATP was hydrolyzed. The enzyme was concentrated up to ~200–220 mg/mL in an Amicon ultrafiltration cell (Model 8010). Protein and nucleotide concentrations were determined spectrophotometrically with $\epsilon_{280}^{mg/mL} = 0.896$ cm^{-1} and a dimer molecular weight of 81 000 (Noda et al., 1954) for the enzyme and $\epsilon_{260}^{mM} = 15.4$ cm^{-1} for ATP and ADP. A Beckman Altex Model 3500 digital pH meter was used for pH measurements.

NMR Measurements. ^{31}P NMR measurements at 121.5 MHz were made on an NTC-300 wide-bore NMR spectrometer equipped with a 12-mm multinuclear probe, a 293C pulse programmer, a Nicolet 1280 computer, and a variable-temperature controller. A typical sample contained ~0.8 mL of the enzyme in an 8-mm o.d. NMR sample tube placed inside a 12-mm NMR tube. D_2O for field-frequency lock was added between the two tubes. 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. A typical stack plot of data obtained for T_1 measurement in an E·CoATP complex is shown in Figure 1. 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 and Experimental Strategy. The theory for the effect of a paramagnetic cation binding on the spin

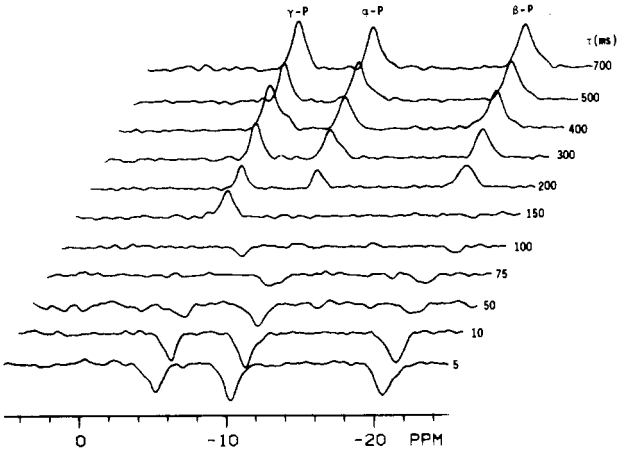

FIGURE 1: A typical T_{1P} measurement at 121.5 MHz for ^{31}P nuclei in ATP bound to creatine kinase in the presence of Co(II) ($p = 0.052$) at $T = 28$ °C. A standard inversion recovery sequence was used with a composite π pulse. NMR parameters: $\pi/2$ pulse width 20.5 μs, sweep width 3012 Hz, data size 2048, line broadening 80 Hz, number of scans 128, and recycle delay 700 ms. Computer fit gives T_{1P} values of 0.20, 0.18, and 0.15 s (SD ~0.01 s) for α-P, β-P, and γ-P, respectively.

relaxation of a ligand nucleus (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen, 1957a,b) has been reviewed extensively (Dwek, 1973; James, 1973; Mildvan & Gupta, 1978; Burton et al., 1979; Jardetzky & Roberts, 1981). Given below is a summary essential for description of the experimental strategy used in this work and for analysis of the data.

The paramagnetic contribution (T_{1P}^{-1}) to the observed relaxation rate ($T_{1,obsd}^{-1}$) is given by

$$T_{1P}^{-1} = T_{1,obsd}^{-1} - T_{1D}^{-1} = p/(T_{1M} + \tau_M) \qquad (2)$$

where T_{1D}^{-1} and T_{1M}^{-1} are the relaxation rates in the diamagnetic and paramagnetic complexes, respectively, τ_M is the lifetime of the paramagnetic complex, and p is the ratio of the cation concentration to that of the ligand. Equation 2 is valid for two exchanging complexes such that $T_{1D}^{-1} \ll T_{1M}^{-1}$ and $p \ll 1$. Clearly, if $\tau_M \gg T_{1M}$, T_{1P}^{-1} is bereft of structural information. Conversely, T_{1M} can be obtained from T_{1P}^{-1} if either $\tau_M \ll T_{1M}$ or $\tau_M \simeq T_{1M}$ and can be quantitatively taken into account. T_{1M} is related to the distance, r, between the cation and the ligand nucleus by

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

in which

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

$$f(\tau_C) = \frac{3\tau_{C1}}{1 + \omega_I^2\tau_{C1}^2} + \frac{7\tau_{C2}}{1 + \omega_S^2\tau_{C2}^2} \qquad (5)$$

and

$$\tau_{Ci}^{-1} = \tau_R^{-1} + \tau_{Si}^{-1} + \tau_M^{-1} \qquad i = 1 \text{ or } 2 \qquad (6)$$

In eq 3–6, S, g, and and ω_S are the spin, the g factor, and the Larmor precession frequency, respectively, of the paramagnetic cation, γ_I and ω_I are the gyromagnetic ratio and the resonance frequency, respectively, of the relaxing nucleus, β is the Bohr magneton, τ_R is the rotational correlation time of the enzyme complex, and τ_{S1} and τ_{S2} are the longitudinal and transverse relaxation times of the cation, respectively. For all the complexes considered here, τ_M does not contribute to eq 6. For Mn(II) complexes, at the magnetic fields used in the present experiments, $\omega_S\tau_{C2} \gg 1$ (McLaughlin et al., 1976), whereas for Co(II) complexes τ_{C1} and τ_{C2} are respectively equal to τ_{S1} and τ_{S2}, and $\omega_I\tau_{C1} \ll 1$ (Melamud & Mildvan, 1975) so that

$$f(\tau_C) = 3\tau_{C1}/(1 + \omega_I^2\tau_{C1}^2) \qquad (7)$$

for Mn(II) complexes and

$$f(\tau_C) = 3\tau_{S1} + 7\tau_{S2}/(1 + \omega_S^2\tau_{S2}^2) \qquad (8)$$

for Co(II) complexes. In eq 3–6 the contribution of hyperfine coupling between the cation and the ligand nucleus is neglected (Brown et al., 1973; Sternlicht et al., 1965), and the following assumptions are implicit: (i) the g factor is isotropic, (ii) the zero field splitting is much smaller than the Zeeman interaction for the cation, and (iii) the electron relaxation is uniquely described by τ_{S1} and τ_{S2}. These assumptions are justifiable for Mn(II) complexes, and τ_{S1} is given by

$$\tau_{S1}^{-1} = B\left[\frac{\tau_V}{1 + \omega_S^2\tau_V^2} + \frac{4\tau_V}{1 + 4\omega_S^2\tau_V^2}\right] \qquad (9)$$

where B is related to the square of the strength of the crystalline field and τ_V is a correlation time for its modulation, provided that $\tau_V \ll \tau_{S1}$ (Abragam, 1961). For Co(II) complexes, however, the above assumptions are questionable, the condition $\tau_V \ll \tau_{S1}$ is not assured, and τ_{S1} and τ_{S2} are not uniquely defined (Bloembergen & Morgan, 1961; Burton et al., 1979).

In light of the above, the experimental strategy for obtaining the quantities of interest for structure determination, T_{1M}^{-1} and $f(\tau_C)$, becomes transparent. Making T_{1P} measurements on enzyme-bound complexes assures conformity with eq 2 and maximizes the contribution of the E·M·S complex. T_{1M} depends on frequency whereas τ_M does not. The activation energies for T_{1M} and τ_M are expected to be in the ranges 1–4 and 5–20 kcal/mol, respectively (Dwek, 1973; Burton et al., 1979). The role of τ_M in T_{1P} is, therefore, determined by making measurements as a function of temperature and frequency and by using two cations with very different paramagnetic effects. When $\tau_M \ll T_{1M}$, the frequency dependence of T_{1P} allows the estimation of $f(\tau_C)$. Line-width measurements will not be used to estimate τ_M (Mildvan & Gupta, 1978; Mildvan et al., 1980) because of the untenable assumptions implicit in the procedure (see later).

RESULTS

Mn(II) Complexes. The values of $(pT_{1P})^{-1}$ obtained for the ^{31}P nuclei in the phosphate groups of MnADP, MnATP, E·MnADP, and E·MnATP and the transition-state analogue complex E·MnADP·NO_3^-·Cre at 20 °C are given in Table I.[2] The data given are based on measurements made at 122 MHz on all the complexes and those at 190 MHz for E·MnADP and E·MnATP complexes. For the enzyme complexes, on the basis of known dissociation constants (Reed et al., 1970), the fractional concentration of the paramagnetic complexes free in solution ([M·S]/[E·M·S]) never exceeds ~3%.

The relaxation rates of α-P and β-P of E·MnADP and those of α-P, β-P, and γ-P of E·MnATP are nearly equal and are unchanged within experimental error between 122 and 190 MHz. This suggests the possibility that $\tau_M \gg T_{1M}$ for the ^{31}P nuclei in these complexes. The drastic reduction in the relaxation rates in the transition-state analogue complex corrobates this view. This complex mimics the transition state because NO_3^- occupies the position of the transferrable

[2] T_{1D} (see eq 2) in diamagnetic enzyme complexes ranged from 1.5 to 3.0 s, and therefore, the contribution of this term was ignored in computing the data given in Table I.

Table I: Paramagnetic Effect $(pT_{1P})^{-1}$ (s^{-1}) of Mn(II) on ^{31}P Relaxation Rates and Corresponding Activation Energies ΔE (kcal/mol) for Various ADP and ATP Complexes Free in Solution and Bound to Creatine 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)
MnADP[b] [ADP, 5.0 mM; $MnCl_2$, 2.5–15 μM]	121.5	5400 ± 300		7200 ± 500			
MnATP[b] [ATP, 4.0 mM; $MnCl_2$, 2.5–15 μM]	121.5	5570 ± 200		7270 ± 200		6930 ± 200	
E·MnADP[b] [enzyme sites, 5.3 mM; ADP, 3.0 mM; $MnCl_2$, 10–50 μM (121.5 MHz) and 20–120 μM (190.2 MHz)]	121.5	1380 ± 100	6.9 ± 0.5	1730 ± 100	6.8 ± 0.5		
	190.2	1330 ± 100		1580 ± 100			
E·MnATP [enzyme sites, 5.3 mM; ATP, 3.5 mM; $MnCl_2$, 34 μM]	121.5	1840 ± 200	6.5 ± 0.5	2170 ± 200	8.1 ± 0.5	2280 ± 200	5.1 ± 0.5
	190.2	2010 ± 200		2210 ± 200		2550 ± 200	
E·MnADP·NO_3^-·Cre [enzyme sites, 5.0 mM; ADP, 2.8 mM; $MgCl_2$, 1.7 mM; $MnCl_2$, 1.15 mM; creatine, 50 mM; KNO_3, 18 mM]	121.5	18 ± 5	17.3 ± 1.0[c]	21 ± 5			

[a] All samples were in 20 mM K-Hepes, pH 8.0. The $(pT_{1P})^{-1}$ values given were measured at 20 °C. The ΔE values were obtained from Arrhenius plots (see Figures 2 and 3) of $(pT_{1P})^{-1}$ in the temperature range 5–35 °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] Measurements were made with four to six values of p = [Mn(II)]/[ADP]. [c] ΔE for β-P was not determined since the signal was too broad at temperatures <20 °C.

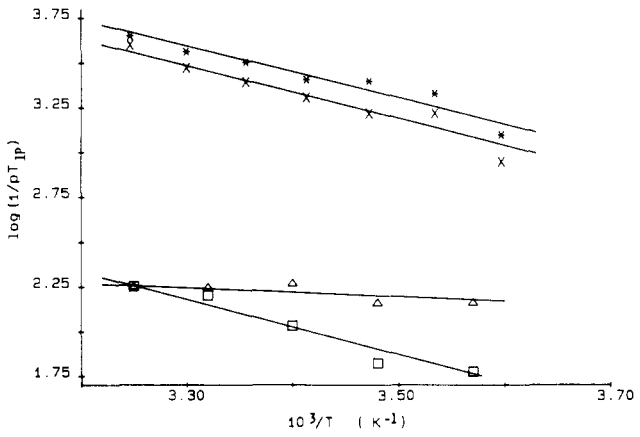

FIGURE 2: log $(pT_{1P})^{-1}$ vs. $10^3/T$ for α-P and β-P of E·MnADP (×, *) and E·CoADP (△, □). Typical sample conditions and activation energies (ΔE) obtained are given in Tables I and II.

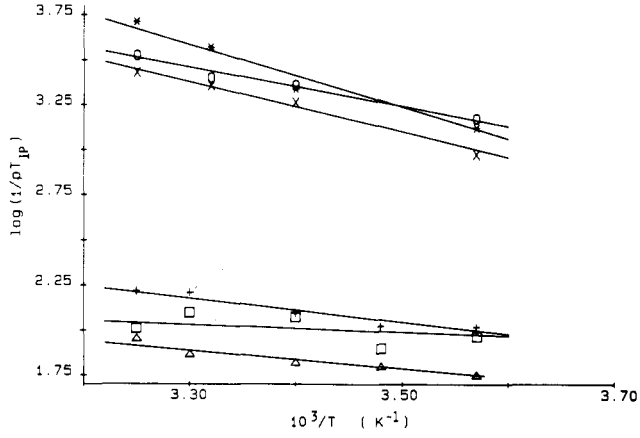

FIGURE 3: log $(pT_{1P})^{-1}$ vs. $10^3/T$ for α-P, β-P, and γ-P of E·MnATP (×, *, ○) and E·CoATP (△, □, +). Typical sample conditions and activation energies (ΔE) obtained are given in Tables I and II.

phosphoryl group (Milner-White & Watts, 1971). Binding of all the components is strengthened at least by 1 order of magnitude in the transition-state analogue complex (Reed & Cohn, 1972; Reed & McLaughlin, 1973), and the lifetimes associated with the complex, such as τ_M, are expected to be similarly longer than those in the lower complexes. A significant increase in τ_M is clearly indicated by the values of $(pT_{1P})^{-1}$ for this complex.

Further evidence for exchange limitation of ^{31}P relaxation times in the Mn(II) complexes is obtained from their temperature dependence in the range 5–35 °C (Figures 2 and 3). If the observed temperature variation were to arise from T_{1M}^{-1}, it can occur if either (i) $\omega_I\tau_{C1} \ll 1$, $\tau_{C1} = \tau_{S1}$, and $\omega_S\tau_V \ll 1$ (see eq 9) or (ii) $\omega_I\tau_{C1} \gg 1$; the first condition is not applicable to Mn(II) complexes, and in the second case a strong frequency dependence of T_{1P} is indicated but was not experimentally observed.[3] Arrhenius plots of $(pT_{1P})^{-1}$ yield activation energies ΔE (see Table I) in the range 5–8 kcal/mol for the Mn(II)–nucleotide complexes (Figures 2 and 3) and 17.3 kcal/mol for the transition-state analogue complex (data not shown). These values of ΔE are much larger than those expected for τ_R or τ_V and are in the range appropriate for τ_M. The marked increase of ΔE in the transition-state analogue complex substantiates the dominant role of τ_M in $(pT_{1P})^{-1}$. Thus, the ^{31}P relaxation data in the Mn(II)–nucleotide and transition-state analogue complexes are determined primarily by the lifetimes of these complexes and not by the distances between the cation and the ^{31}P nuclei.

Comparison with Experiments Using Excess Substrate Concentrations. It is instructive to compute, using the data in Table I and in Figures 2 and 3, the expected results if the experiments were performed with substrate concentrations in excess of the enzyme. Assuming a sample composition such that [MnADP]:[E·MnADP] = 3:1 and p = 0.01, noting that $(pT_{1P})^{-1}$ is ~7200 s^{-1} for MnADP and ~1600 s^{-1} for E·MnADP, T_{1P}^{-1} of the sample is 58 s^{-1}, out of which 4 s^{-1} is contributed by E·MnADP and the rest by MnADP. The relaxation rate of interest is thus obtained with dubious accuracy as a small difference of two relatively large quantities.[4] Furthermore, since $(pT_{1P})^{-1}$ of E·MnADP increases from ~1000 s^{-1} at 5 °C to ~4000 s^{-1} at 35 °C whereas that for MnADP is virtually unchanged, if the temperature variation were measured with the above sample, T_{1P}^{-1} would have varied

[3] The increase of $(pT_{1P})^{-1}$ with temperature is not due to a possible increase in free MnADP. At 24 °C, [MnADP]/[E·MnADP] is <3%. If this value were to double in the range 5–35 °C (which is unlikely), the additional contribution to the observed $(pT_{1P})^{-1}$ is <200 s^{-1}. Note, further, that if $\tau_M \gg T_{1M}$, nearly the same value of $(pT_{1P})^{-1}$ and ΔE should be obtained for the different nuclei. The spread in these values arises from factors such as the contribution to the relaxation rate from free Mn–nucleotide in the sample and the accuracy of the T_{1P} measurements.

[4] This problem is particularly acute for substrates such as ADP and ATP to which the cation binds free in solution as well as on the enzyme, and the value of T_{1M}^{-1} in the free complex is larger than that in the bound complex by a significant factor (see Table I).

from 56.5 s^{-1} at 5 °C to 64 s^{-1} at 35 °C. This change is barely outside experimental error, and such a measurement would have led to the conclusion that $(pT_{1P})^{-1}$ was not exchange limited.

Another procedure, often employed in previous studies to infer fast exchange, was to use line-width measurements to determine $(pT_{2P})^{-1}$ assuming relations similar to eq 2 to hold and to invoke the argument that since $(pT_{2P}) \geq \tau_M$ and $T_{1M} \geq T_{2M}$, if $T_{1P} \gg T_{2P}$ then $T_{1M} \gg \tau_M$. Such a procedure implicitly assumes that the resonances in the enzyme complex are Lorentzian with line widths arising exclusively from relaxation processes governing T_{1M}. Therefore, other factors that contribute to the line width such as spin multiplicity of the resonances (Vasavada et al., 1980), chemical shifts caused by cation binding, possible conformational heterogeneity, and exchange processes between such chemically shifted resonances are deemed to be absent. The ^{31}P resonances of different diamagnetic enzyme-bound complexes show rather broad non-Lorentzian lines arising from factors such as the above (Nageswara Rao & Cohn, 1981). For example, from the line width of α-P of E·MnADP, $(pT_{2P})^{-1} \simeq 8000\ s^{-1} \gg (pT_{1P})^{-1} \simeq 1600\ s^{-1}$ (Table I). Similarly, for γ-P of E·MnATP, $(pT_{2P})^{-1} \simeq 10^4\ s^{-1} \gg (pT_{1P})^{-1} = 2280\ s^{-1}$. However, as shown above, T_{1P} is exchange limited in these complexes. The criterion $(pT_{2P}) \geq \tau_M$ based on line-width measurements, therefore, leads to a grossly incorrect estimate of τ_M.

Co(II) Complexes. Values of $(pT_{1P})^{-1}$ obtained for ^{31}P nuclei in the phosphate groups of CoADP, CoATP, E·CoADP, E·CoATP, and E·CoADP·NO_3^-·Cre at 30 °C are given in Table II. For all but the transition-state analogue complex, measurements were made at three frequencies, viz., 81, 122, and 190 MHz. $(pT_{1P})^{-1}$ for CoADP showed a dependence on ADP concentration, and therefore, the values at high (5 mM) and low (0.45 mM) concentrations along with those obtained by extrapolation to infinite dilution are given. The data for CoATP do not show a concentration dependence in the same range. For the three enzyme-bound complexes, the temperature dependence of $(pT_{1P})^{-1}$ at 122 MHz was measured in the range 5–35 °C. Arrhenius plots for E·CoADP and E·CoATP are included in Figures 2 and 3, respectively. The values of ΔE are given in parentheses in Table II.

Figure 2 shows a striking difference in the temperature variation of $(pT_{1P})^{-1}$ for α-P and β-P of E·CoADP with ΔE values of 1.2 and 7.1 kcal/mol, respectively. A marked temperature dependence of the line shape of β-P (MgADP) was observed in the range 5–25 °C (Nageswara Rao & Cohn, 1981) for the enzyme complexes of creatine kinase and was interpreted in terms of a conformational heterogeneity that is averaged by exchange at higher temperatures. The line shape of α-P (MgADP), in contrast, is not significantly altered. The greater temperature dependence of $(pT_{1P})^{-1}$ of β-P compared to that of α-P is thus attributable to chemical exchange between the heterogeneous conformations in the enzyme-bound complex rather than to τ_M; for, if τ_M were to dominate, nearly the same ΔE should be obtained for both (see data for E·MnADP complex in Table I). The ΔE value of 1.2 kcal/mol for α-P characterizes the temperature dependence arising from $f(\tau_C)$. All the T_{1P} measurements used for structure determination were made at 30 °C where the value of T_{1M} for β-P stabilizes due to chemical exchange and shows diminished sensitivity to temperature change (see Figure 2).

Note that the $(pT_{1P})^{-1}$ data for E·CoATP as a function of temperature (Figure 3) do not display effects of the conformational heterogeneity discussed above. This disparity also occurred for the corresponding diamagnetic complexes (Na-

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

complex (sample composition)	$(pT_{1P})^{-1}$ (s^{-1}) and ΔE (kcal/mol) (in parentheses)									
	81 MHz			121.5 MHz			190.2 MHz			
	α-P	β-P	γ-P	α-P	β-P	γ-P	α-P	β-P	γ-P
CoADP (ADP, 5.0 mM; $CoCl_2$, 25–150 μM)	290 ± 15	2200 ± 150		115 ± 10	1140 ± 80		170 ± 10	1580 ± 80	
CoADP (ADP, 0.45 mM; $CoCl_2$, 4.2 μM)	270 ± 15	810 ± 150		265 ± 20	630 ± 50		260 ± 15	590 ± 50	
CoADP (extrapolated values)[b]				280 ± 20	560 ± 50				
CoATP (ATP, 2.7 mM; $CoCl_2$, 15–125 μM)	150 ± 20	210 ± 20	250 ± 20	120 ± 20	150 ± 20	210 ± 20	135 ± 20	160 ± 20	225 ± 20
E·CoADP (enzyme sites, 4.9 mM; ADP, 2.7 mM; $CoCl_2$, 120–470 μM)	270 ± 25	250 ± 20		115 ± 20 (1.2 ± 0.2)	80 ± 20 (7.1 ± 0.5)		290 ± 25	160 ± 15	
E·CoATP (enzyme sites, 5.0 mM; ATP, 3.4 mM; $CoCl_2$, 180–520 μM)	230 ± 30	250 ± 40	220 ± 30	85 ± 20 (2.4 ± 0.2)	120 ± 20 (1.0 ± 0.2)	170 ± 20 (3.1 ± 0.2)	170 ± 20	260 ± 40	265 ± 30
E·CoADP·NO_3^-·Cre (enzyme sites, 5.6 mM; ADP 3.2 mM; $CoCl_2$, 0.93 mM; creatine, 43 mM; KNO_3, 47 mM)				60 ± 10 (17.2 ± 1.0)	65 ± 10 (19.1 ± 1.0)				

[a] All samples were buffered with 20 mM K-Hepes, pH 8.0. The $(pT_{1P})^{-1}$ values given were measured at 30 °C with at least four different values of p = [Co(II)]/[ADP]. Measurements were made at 81, 121.5, and 190.2 MHz. The ΔE values, given in parentheses, were obtained at 121.5 MHz from Arrhenius plots (see Figures 2 and 3) of $(pT_{1P})^{-1}$ in the temperature range 5–35 °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] At infinite dilution (see text).

geswara Rao & Cohn, 1981). The ΔE values in E·CoATP are in the range 1–3 kcal/mol similar to that for α-P (E·CoADP) and significantly lower than those for the Mn(II) complexes. Furthermore, the $(pT_{1P})^{-1}$ values for both E·CoADP and E·CoATP complexes at 30 °C show a moderate but reproducible frequency dependence outside experimental error. The relaxation rates in the Co(II) complexes are, therefore, primarily determined by T_{1M}, and the contribution of τ_M is much smaller in comparison.

For the transition-state analogue complex E·CoADP·NO_3^-·Cre, however, the values of ΔE are 17.2 and 19.1 kcal/mol for α-P and β-P, respectively, similar to that for the Mn(II) complex (Table I), indicating that $(pT_{1P})^{-1}$ is significantly contributed by exchange. Considering the average value of $(pT_{1P})^{-1}$ = 16 ms for α-P and β-P at 30 °C in the Co(II) complex (Table II) and assuming ~10 ms of this (average T_{1M} value for the E·CoADP complex) is T_{1M} yield 6 ms as an estimate of τ_M. In going from E·MnADP complex to E·MnADP·NO_3^-·Cre, τ_M [which equals pT_{1P} for the Mn(II) complexes] increases by a factor of ~75. Tacitly assuming a similar increase for the corresponding Co(II) complexes suggests that for the E·CoADP complex $\tau_M \sim 0.1$ ms.[5] This estimate is qualitatively reasonable although it is not likely to be numerically accurate. Nevertheless, it clearly shows that the $(pT_{1P})^{-1}$ data for the E·CoADP and E·CoATP complexes may be used to obtain structural information.

Calculation of Distances. The calculation of Co(II)–^{31}P distances is beset with theoretical problems related to $f(\tau_C)$ in these complexes. Some theoretical approaches to obviate these difficulties were recently suggested by Benetis et al. (1983). Their method is yet to be applied to relaxation processes appropriate for the Co(II) complexes of interest here. In addition, the anisotropic g tensor of Co(II) (Sternlicht, 1965) and the likelihood of multiple electron relaxation rates (Rubinstein et al., 1971) introduce complications that are not insignificant although they are tractable once a correct theoretical procedure for the calculation of spectral densities is established.

However, previous relaxation measurements on Co(II) complexes of various enzymes indicate values of $f(\tau_C)$ in the range 5×10^{-13} to 5×10^{-11} s (Mildvan et al., 1980; Villafranca, 1984). Furthermore, the $(pT_{1P})^{-1}$ data for the enzyme-bound Co(II) complexes (Table II) at 81, 121.5, and 190.2 MHz exhibit a frequency dependence with a minimum at 121.5 MHz. [The corresponding electron resonance frequencies (ω_S) for g = 4.33 (see below) are 1.7×10^{12}, 2.7×10^{12}, and 4.2×10^{12} rad s^{-1}.] A similar frequency dependence was also observed for CoADP at high ADP concentrations. The concentration dependence of $(pT_{1P})^{-1}$ in CoADP indicates intermolecular interactions that reduce accessibility of the immediate environment of Co(II) to the solvent and thereby increase τ_V. The similarity in the frequency dependence of $(pT_{1P})^{-1}$ in E·CoADP and E·CoATP with the above is consistent with this interpretation since Co(II) in the enzyme complexes is less accessible to solvent than it is in free nucleotide complexes. Thus at the elevated values of τ_V appropriate for the enzyme-bound complexes $f(\tau_C)$ becomes frequency dependent. On the basis of the qualitative features of eq 8 and 9 and noting that $f(\tau_C)$ has a minimum at $\omega_S^{-1} \simeq 3.7 \times 10^{-13}$ s, it is plausible to choose the range 10^{-12} s < $f(\tau_C)$ < 5×10^{-12} s (for the measurements at 121.5 MHz).

Table III: Distances of ^{31}P Nuclei from the Cation [Mn(II) or Co(II)] in Various Metal–Nucleotide Complexes Free in Solution and Bound to Creatine Kinase[a]

complex	$f(\tau_C)$ or τ_C	cation–^{31}P distance (Å)		
		α-P	β-P	γ-P
CoADP	$f(\tau_C) = 10^{-12}$ to 5×10^{-12} s	2.6–3.5	2.4–3.1	
CoATP	$f(\tau_C) = 10^{-12}$ to 5×10^{-12} s	3.0–4.0	2.9–3.8	2.7–3.6
E·CoADP	$f(\tau_C) = 10^{-12}$ to 5×10^{-12} s	3.0–4.0	3.3–4.3	
E·CoATP	$f(\tau_C) = 10^{-12}$ to 5×10^{-12} s	3.2–4.2	3.0–4.0	2.9–3.8
MnADP	$\tau_C = 10^{-10}$ s	3.7	3.5	
MnATP	$\tau_C = 10^{-10}$ s	3.7	3.55	3.55
E·MnADP	$\tau_C = 10^{-9}$ s (lower limit)	6.4[b]	6.2[b]	
E·MnATP	$\tau_C = 10^{-9}$ s (lower limit)	6.0[b]	5.9[b]	5.8[b]

[a] Calculations are based on eq 4, with C = 675 and 601 Å s$^{-1/3}$, respectively, for Co(II) and Mn(II) complexes, using $(pT_{1P})^{-1}$ data at 121.5 MHz (see Tables I and II). The distances in Co(II) complexes are given as a range corresponding to the range chosen for $f(\tau_C)$ (see text). Errors arising from $(pT_{1P})^{-1}$ are ~2–4%. Most of the uncertainty in the distances is due to the estimation of $f(\tau_C)$. [b] Lower limits of distances obtained if $(pT_{1P})^{-1}$ data at 20 °C (Table I) for E·MnADP and E·MnATP are *incorrectly* considered not to be exchange limited.

For C in eq 4 we use $S = {}^3/_2$ and a value of 4.33 for the isotropic g factor (Abragam & Pryce, 1970; Fung et al., 1974; Melamud & Mildvan, 1975), leading to C = 675 Å s$^{-1/3}$. The EPR spectrum of E·CoADP·$HCOO^-$·Cre at 4.2 K indicates an anisotropic g tensor (McLaughlin et al., 1976). Adequate information is not available to evaluate the contribution of this anisotropy to eq 3. Nevertheless, since the Co(II)–^{31}P distances are calculated for a range in $f(\tau_C)$, it may be expected that the correction due to the g anisotropy is absorbed in the same range.[6] The distances calculated on this basis are given in Table III.

Mn(II)–^{31}P distances in MnADP and MnATP complexes, calculated with C = 601 Å s$^{-1/3}$ (i.e., g = 2.0 and $S = {}^5/_2$) and a correlation time of 10^{-10} s (Sloan & Mildvan, 1976), are also listed in Table III. These distances agree with previous determinations (Brown et al., 1973; Sloan & Mildvan, 1976) and indicate direct coordination of Mn(II) with the phosphate groups. The general agreement between these and the Co(II)–^{31}P distances in CoADP and CoATP indicates that the range estimated above for $f(\tau_C)$ is correct.

The Co(II)–^{31}P distances obtained for E·CoADP and E·CoATP complexes signify direct coordination of Co(II) with all the phosphate groups. A second coordination sphere is far outside the range and can be safely ruled out. The conclusion is in agreement with Mn(II) EPR results of Leyh et al. (1984) and Reed & Leyh (1980). The anisotropic g tensor in the Co(II) complexes introduces angular factors in eq 3 that depend on the orientation of the Co(II)–^{31}P vector in the principal axis system of the g tensor. Leyh et al. (1985) propose that in E·MnATP the three phosphate groups are located so as to yield a 3-fold axis of symmetry passing through Mn(II). Assuming the same structure for the Co(II) complex implies identical angular factors in the expressions for T_{1M}^{-1} of the three ^{31}P nuclei.[6] Thus, in spite of the g anisotropy the ratios of T_{1M} values for the three ^{31}P nuclei are equal to the sixth power of the ratios of the corresponding Co(II)–^{31}P distances

[5] This value is shorter than $\tau_M \sim 0.6$ ms for E·MnADP and 0.4 ms for E·MnATP (see Table I). T-jump measurements of the dissociation rates of Mn(II) and Co(II) from their ATP complexes yield lifetimes < 0.1 and 0.5 ms, respectively (Hammes & Levison, 1964). The reversal in the relative values of the lifetimes of the complexes on the enzyme indicates that τ_M values obtained for the E·M·S complexes are not primarily determined by the lifetimes of M·S complexes of the two cations. The governing steps for τ_M may well be different for the two cations.

[6] On the basis of molecular models of this chelate structure, an angle of ~50° is subtended between the symmetry axis and the cation–^{31}P dipolar vector (G. H. Reed, private communication). For an axially symmetric g tensor, with $|g_\parallel - g_\perp|/g_{isotropic} = 0.5$ ($g_\parallel$ is the principal value along the symmetry axis), the correction to T_{1M}^{-1} for this orientation is ~2.5% if $g_\parallel < g_\perp$ and 10.5% if $g_\parallel > g_\perp$ (K. V. Vasavada and B. D. Nageswara Rao, unpublished calculations).

to a good approximation. From the $(pT_{1P})^{-1}$ values (Table II), it is evident that these three distances are equal within experimental error.

If the $(pT_{1P})^{-1}$ data for E·MnADP and E·MnATP complexes were erroneously construed not to be exchange limited, assuming a lower limit of 10^{-9} for τ_C (McLaughlin et al., 1976; Reed & Leyh, 1980) yields a set of minimum values for Mn(II)–^{31}P distances (5.8–6.5 Å) given at the bottom of Table III. All these distances are appropriate for a second coordination sphere.[7] It may be recalled that such an indirect coordination between Mn(II) and ATP was concluded for the enzyme complexes of pyruvate kinase (Sloan & Mildvan, 1976).

Discussion

It has been observed that a useful feature of the paramagnetic relaxation method for structure determination is that because of the r^6 dependence of T_{1M}, errors are scaled down in the calculation of r. This is clearly true for errors in the estimation of C and $f(\tau_C)$ in eq 3 and for those in an unambiguous determination of T_{1M}. However, if τ_M is comparable or larger than T_{1M} and the experimental protocol and procedure are not incisive enough to recognize that to be the case, the value of r obtained will not be of any significance in spite of the presence of r^6 in eq 3. The experimental strategy adapted in the present work to determine the contribution of τ_M to the observed relaxation rates and the results obtained illustrate that the validity of fast exchange conditions should be ascertained with considerable deliberation. Making measurements on enzyme-bound substrates clearly maximizes the contribution of the E·M·S complex to T_{1P}^{-1} and provides the accuracy necessary for reliable interpretation of the variations thereof. Furthermore, the following points of methodological relevance emerge from the results: (i) The temperature dependence of T_{1P}^{-1} is useful in providing a qualitative separation of the roles of τ_M and T_{1M} only if the variation in T_{1M} arises exclusively from $f(\tau_C)$. However, if other factors such as conformational changes in the enzyme complexes contribute, as in the case of the measurements on E·CoADP, the results should be interpreted with caution. (ii) The estimates of τ_M in Mn(II) and Co(II) complexes indicate that τ_M may be limited by different steps for the two cations. This result is not altogether surprising but should not be overlooked if the choice of the cation were to be used to control τ_M. (iii) Perhaps the most important point with reference to the procedure employed in a number of previous studies is that the use of line-width measurements to determine T_{2P} and estimate τ_M through the criterion $(pT_{2P}) \geq \tau_M$ is based on assumptions that are easily violated and, therefore, leads to incorrect results.

The Co(II)–^{31}P distances obtained imply that the cation is directly coordinated to the nucleotide on the enzyme as it is off the enzyme. Preliminary results obtained with lobster muscle arginine kinase indicate a similar pattern (B. D. Ray, G. K. Jarori, and B. D. Nageswara Rao, unpublished experiments). These results are at variance with the previous conclusion of second-hydration-sphere coordination of Mn(II)–nucleotide complexes of pyruvate kinase (Sloan & Mildvan, 1976). It is noteworthy that if their methodology were used for the creatine kinase experiments, the exchange limitation in the Mn(II) complexes would not have been recognized and the Mn(II)–^{31}P distances thus obtained would have been appropriate for a second-coordination sphere.

The conclusion of direct coordination between the cation and the enzyme-bound nucleotides is in agreement with that obtained from Mn(II) EPR studies of a transition-state analogue complex (Reed & Leyh, 1980) and of an E·MnATP complex in the presence of a substrate analogue of creatine (Leyh et al., 1985). The EPR results, however, do not contain unambiguous evidence that the E·MnATP complex is tridentate, nor are the distances in the structure calculable from the data. The ^{31}P relaxation rates in E·CoATP show that this complex is tridentate, with the distances of the three ^{31}P nuclei from the cation nearly equal to each other, consistent with the 3-fold axis of symmetry passing through the cation suggested by the EPR study. The NMR experiments are, however, unable to probe the structure of the transition-state analogue complex because of the long lifetimes (τ_M) of these complexes compared to the expected values of T_{1M} even in the presence of Co(II). The results of EPR and NMR measurements thus complement each other in a useful fashion.

The T_{1P} data obtained with the Mn(II) complexes of creatine kinase and the τ_M values estimated show that for these complexes T_{1M} will be larger than τ_M when the cation–^{31}P distances are larger than ~6.5 Å. On the other hand, for the Co(II) complexes, paramagnetic effects on relaxation will be too small to be accurately measurable for distances greater than 6.5 Å. Thus, Co(II) and Mn(II) can be used in a complementary manner to obtain structure-dependent relaxation data in two different ranges of cation–substrate nucleus distances. Analysis of the Co(II) data, however, is beset with uncertainties in $f(\tau_C)$, and some theoretical progress in obtaining a reliable estimate of $f(\tau_C)$ on the basis of the observed frequency dependence of T_{1M} will be valuable. Nevertheless, ^{13}C and ^{15}N nuclear spin-relaxation measurements on specifically labeled nucleotides bound to creatine kinase are likely to provide additional valuable structural information on these complexes. Such experiments are in progress.

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Registry No. MnADP, 69828-68-8; MnATP, 56842-80-9; CoADP, 78969-59-2; CoATP, 18925-85-4.

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[7] Conversely, for r = 3–3.5 Å, appropriate for first coordination sphere, with $\tau_C = 10^{-9}$ s, T_{1M} = 8–20 μs [for Mn(II) complexes] whereas the experimental values of pT_{1P} are in the range 0.4–0.8 ms (see Table I) for E·MnADP and E·MnATP. Thus, for these complexes $pT_{1P} = \tau_M \gg T_{1M}$.

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