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Paramagnetic Effects on Nuclear Relaxation in Enzyme-Bound Co(II)–Adenine Nucleotide Complexes: Relative Contributions of Dipolar and Scalar Interactions

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^{31}P NMR measurements on CoADP bound to creatine kinase designed to estimate the relative contribution of scalar and dipolar interactions to ^{31}P spin relaxation rates show that these rates are primarily due to distance-dependent dipolar interactions and that the contribution of the scalar interaction is negligible. © 1999 Academic Press

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In vivo, enzymatic reactions involving adenosine 5′-triphosphate (ATP) use Mg(II) for activation. *In vitro,* Mg(II) may be replaced by paramagnetic cations Mn(II) or Co(II). This feature led to the possibility of structural characterization of enzyme-bound reaction complexes through measurements of distance-dependent enhancement of spin relaxation rates (T_{1M}^{-1}) of nuclei in the vicinity of the paramagnetic cation. Such measurements are performed under sample conditions in which the enzyme · metal · substrate (E · M · S) and enzyme · substrate (E · S) complexes are undergoing chemical exchange, leading to the circumstance that the observed relaxation rates are distance dependent only if $T_{1M} \gg \tau_M$, where τ_M is the lifetime of E · M · S. Previous work has shown that, for typical values of τ_M, T_{1M} for Mn(II)-complexes satisfy this condition only for cation–nucleus distances greater than ~6.5 Å, and for shorter distances of ~2.0 to 4.0 Å, it is necessary to use Co(II) as the activating cation (*1–4*).

Nuclear spin relaxation enhancement by paramagnetic cations arises due to electron–nucleus dipolar interactions ($\mathbf{I} \cdot \underline{\mathbf{D}} \cdot \mathbf{S}$) and a scalar interaction ($A\mathbf{I} \cdot \mathbf{S}$) which represents contact and, if the g tensor is anisotropic, pseudo-contact interactions. The scalar interaction also gives rise to a shift of the nuclear resonance, contact (or pseudo-contact) shift, by effectively adding a term $AI_Z\langle S_Z\rangle$ to the nuclear spin Hamiltonian where $g\beta\langle S_Z\rangle$ is the thermal equilibrium magnetization of the cation. A measurement of this shift, therefore, allows an estimate of the scalar contribution to the observed relaxation rate. Since the relaxation rate due to dipolar interaction depends on the cation–nucleus distance, whereas that due to contact interaction does not, an estimate of the latter contribution is useful for evaluating the distance data computed from paramagnetic relaxation measurements. This question is relevant for atoms directly coordinated to the cation, and those connected to the coordinated atom, so that the cation–nucleus distances are short (<4 Å), as in the case of Co(II)–^{31}P distances in E · CoATP or E · CoADP. The published Co(II)–^{31}P distances in enzyme–nucleotide complexes were calculated by ignoring possible contributions of the scalar interaction (*1–4*). This procedure seemed reasonable because the distances obtained for the complexes of creatine kinase (*1*) and 3-phosphoglycerate kinase (*2*), for example, are consistent with the direct chelation of the cation with all phosphate groups of ATP (or ADP), which was previously demonstrated by the observation of ^{17}O superhyperfine interactions with Mn(II) as seen in Mn(II) EPR (*5, 6*). However, the contribution of the scalar interaction to the ^{31}P spin relaxation in the presence of Co(II) has not been explicitly estimated by a direct measurement of the contact shift for the complexes of any of these enzymes. This Communication reports such a measurement for CoADP bound to creatine kinase.

Initial attempts to record the ^{31}P NMR spectrum by adding Co(II) to an aqueous solution of E · ADP such that [Co(II)]/[ADP] = p (<1) have resulted in a reduction in the observed ^{31}P signal (relative to that in the absence of the cation) by a fraction approximately equal to p (for small values of p), indicating that most of the signal from the cation-bound complex is not observed. Searches for this signal over a range of about ±2500 ppm have been unsuccessful in detecting any other resonances. The bound E · CoADP signal is either too broad to be observable (probably due to exchange) or too far outside the chemical-shift range scanned. In order to overcome the first of these possibilities, it was planned to make the measurements at low temperatures (<0°C) in appropriate cryosolvents. A solvent containing ethylene glycol in 0.2 M K-Hepes buffer, pH 8.2, was found to be useful for this purpose. By varying the amount of ethylene glycol, it was possible to

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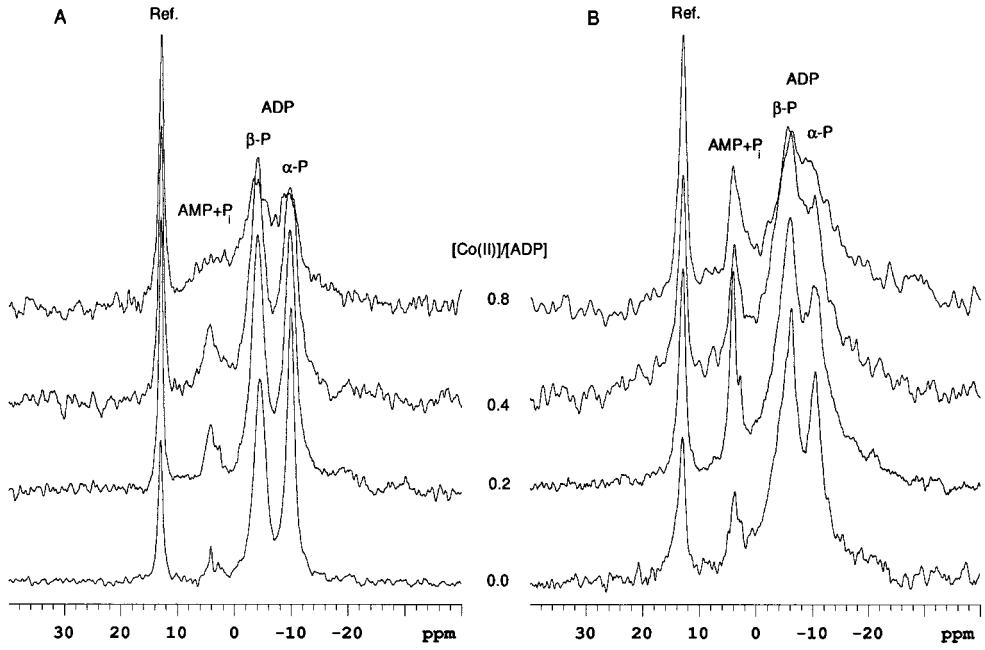

FIG. 1. ^{31}P NMR spectra (120 MHz, Varian Unity 300) of E · ADP at different added Co(II) concentrations. [Co(II)]:[ADP] ratios are noted and ^{31}P signals are labeled. Typical sample consisted of ~1.2 ml of E · ADP in an 8-mm NMR tube held concentrically inside another 10-mm tube. The annulus contained a ^{31}P-concentration and chemical-shift reference and field-frequency lock solution whose relaxation is enhanced by adding $MnCl_2$. Sample composition: (A) 5.9 mM creatine kinase active sites, and 4.6 mM ADP, in 200 mM K-Hepes, pH 8.2, 5°C; reference solution, 4 mM phenylphosphonate, 40 μM $MnCl_2$, pH 7.1, in D_2O. (B) Same as A except 6.9 mM creatine kinase active sites, 4.9 mM ADP, −5°C in 30% ethylene glycol solutions. NMR parameters: sweep width, 48,019 Hz; data size, 8192; line broadening, 50 Hz; number of scans, 2000; recycle delay, 3 s; 30° pulse width, 8 μs.

reduce the temperature down as far as −30°C. Some optimization was necessary to balance the various causes of line broadening such as exchange rates and solvent viscosities in order to record the signals and integrate their intensities with sufficient accuracy. Before the measurements were begun, the enzyme was assayed for activity in the cryosolvent mixture. The activity of creatine kinase at 21°C measured in a standard assay mix (*7*) in aqueous solution at pH 9.0 with Mg(II) as the activating cation is 57 IU; with Co(II) it is reduced to 12% of this value. Under the same conditions but in 30% ethylene glycol solution, the activity was reduced to 35% with Mg(II) and to 4% with Co(II). The activity was measured at 21°C, although the signals were recorded at −5°C (see below) because it is too low to measure at −5°C. In addition, it was ascertained that the enzyme was not irreversibly denatured when the temperature was lowered by measuring the activity once again (at 21°C) after the experiment.

Figure 1A shows the ^{31}P spectrum of E · CoADP in aqueous solution at 5°C, and Fig. 1B shows those in the cryosolvent at −5°C. Experimental details are given in the figure legend. Under the sample conditions chosen, on the basis of previously published data on dissociation constants (*1, 8, 9*), over 95% of the nucleotide is present in the enzyme–bound form, and over 88% of the Co(II) is present as E · CoADP. In the presence of 30% ethylene glycol (Fig. 1B), measurements of activation energy of ^{31}P relaxation rates (see below) indicate that the binding of the CoADP to the enzyme is tighter than in the aqueous solution. The different ^{31}P signals seen in the spectra and the ratios of [Co(II)]:[ADP] used are shown in Fig. 1. There was some accumulation of P_i (and AMP) due to a small amount of unavoidable enzymatic hydrolysis of ADP during the course of the experiment. This hydrolysis limited the duration of signal averaging in each of the spectra shown in Fig. 1. The ratios of the integrated intensities of the ^{31}P signals of all the phosphate groups of ADP (including P_i and AMP) to that of the phenylphosphonate standard for both samples are shown in Table 1. It is evident from the data on the aqueous solution (Fig. 1A) that for $p \leq 0.4$ the fractional reduction in signal intensity is approximately equal to p (note that [E · CoADP] is about 10% less than [Co(II)] as stated above), indicating that Co(II)-bound complexes do not contribute to the observed resonances. For $p = 0.8$, the reduction in signal intensity is only ~50%. Several factors may have contributed to this change at higher Co(II) concentrations: (1) there may be some contribution to the signal intensity from the broad signal (see below) due to the larger fraction of E · CoADP; (2) the accuracy of integration of the enzyme-bound signals is lower due to lower signal-to-noise and to nucleotide hydrolysis; and (3) adventitious binding of the cation to the protein. On the other hand, in the data for the 30% ethylene glycol solution (Fig. 1B), the total signal intensity remains unchanged within experimental error, clearly indicating that E · CoADP signals are observed. Activation energies of the paramagnetic relaxation rates $(T_{1P})^{-1}$ of ^{31}P nuclei, determined in the temperature

TABLE 1

Ratios of Integrated ^{31}P NMR Signal Intensities of the Total Phosphates in E · ADP for Different Added Co(II) Concentrations to That of Phenylphosphonate Reference for Two Sample Conditions[a]

[Co(II)]/[ADP]	Area (phosphates)/area (reference)	
	Aqueous solution	30% ethylene glycol
0	7.0	4.9
0.2	6.1	5.4
0.4	4.4	5.6
0.8	3.8	4.8

Note. Experimental error, estimated based on multiple measurements, is about ±10%.

[a] Sample conditions: (1) In aqueous solution at 5°C and (2) in 30% ethylene glycol solution at −5°C, both with 0.2 M K-Hepes at pH 8.2.

range −5 to 15°C, yielded 11.4 kcal/mol for α-P(ADP) and 9.8 kcal/mol for β-P(ADP), indicating that the slow exchange condition ($\tau_M > T_{1M}$) prevails in the cryosolvent. The activation energy in aqueous solutions was 1–3 kcal/mol, appropriate for fast exchange (*1*). Thus, the exchange broadening is reduced in the cryosolvent making the E · CoADP signals observable and the signal intensity remains unchanged as Co(II) is added. It is evident from Fig. 1B that the ^{31}P chemical shifts of E · CoADP are within the range of the recorded spectra, i.e., less than ±50 ppm.

The contribution of dipolar and scalar interactions $(T_{1D})^{-1}$ and $(T_{1C})^{-1}$ to $(T_{1M})^{-1}$ can be estimated on the basis of well-known expressions for these relaxation rates (*10, 11*), written assuming an isotropic g tensor, and isotropic rotational tumbling as

$$(T_{1M})^{-1} = (T_{1D})^{-1} + (T_{1C})^{-1}, \quad [1]$$

$$(T_{1D})^{-1} = \frac{2}{15}\frac{g^2\beta^2\gamma_I^2 S(S+1)}{r^6}\left[\frac{3\tau_{C1}}{1+\omega_I^2\tau_{C1}^2} + \frac{7\tau_{C2}}{1+\omega_S^2\tau_{C2}^2}\right], \quad [2]$$

$$(T_{1C})^{-1} = \frac{2}{3}A^2 S(S+1)\left[\frac{\tau_{S2}}{1+\omega_S^2\tau_{S2}^2}\right], \quad [3]$$

with

$$\tau_{Ci}^{-1} = \tau_R^{-1} + \tau_{Si}^{-1}, \quad i = 1, 2, \quad [4]$$

where τ_{Si} (i = 1, 2) are the electron relaxation times of the cation, τ_R is the rotational correlation time, and the other symbols have their usual meaning. The contact shift, δ_C, is given by

$$\delta_C = A\left(\frac{g\beta}{\gamma_I}\right)\frac{S(S+1)}{3kT}. \quad [5]$$

For ^{31}P relaxation in the Co(II)–nucleotide complexes (free and enzyme-bound), with a Co(II)–^{31}P distance of 3 Å, T = 268 K, and EPR parameters appropriate for Co(II) (*1, 10, 11*), g = 4.33, $S = \frac{3}{2}$, and $\tau_{S1} \approx \tau_{S2} \approx \tau_S \approx 10^{-12}$ s ($\therefore \tau_{C1} = \tau_{C2} = \tau_S$ and $\omega_I\tau_S \ll 1$), we find that the ratio of the contact and dipolar contributions to T_{1M}^{-1} is given by

$$\xi = \frac{(T_{1C})^{-1}}{(T_{1D})^{-1}} = 1.2291 \times 10^6 \frac{\delta_C^2}{10 + 3(\omega_S\tau_S)^2}. \quad [6]$$

At a magnetic field corresponding to a ^{31}P NMR frequency of 120 MHz, $\omega_S = 2.7 \times 10^{12}$ rad/s, so that

$$\xi(120\ \text{MHz}) = 3.8566 \times 10^4\delta_C^2,$$

and with δ_C = 50 ppm,

$$\xi(120\ \text{MHz}, 50\ \text{ppm}) = 96.42 \times 10^{-6}. \quad [7]$$

Thus the contribution of the contact interaction to T_{1M}^{-1} is vanishingly small compared to that of the dipolar interaction. There could be some uncertainty in the value of τ_S. It is generally accepted that this value lies in a range around 10^{-12} s (*1, 10, 11*). Equation [6] shows that as a function of the operating frequency, the maximum value of ξ, $\xi_{max} = 1.2291 \times 10^5\delta_C^2$, occurs for $\omega_S\tau_S \ll 1$. Furthermore, it is important to recognize that at higher magnetic fields, such that $\omega_S\tau_S > 1$, $(T_{1C})^{-1}$ decreases whereas $(T_{1D})^{-1}$ reaches a constant value as seen from Eqs. [1], [2], and [6], so that the value of ξ is reduced. Alternatively, with $\omega_S\tau_S$ = 2.7 as in Eq. [7], $(T_{1C})^{-1}$ will be comparable to $(T_{1D})^{-1}$, i.e. ξ = 1, only for δ_C = 5092 ppm which is inordinately large.

The estimates made above do not include contributions from anisotropy of the g factor. The Co(II) EPR spectrum of the creatine kinase transition state analog complex E · CoADP · COO^- · creatine, recorded at 4.2 K, displays g anisotropy with principal g-tensor values in the neighborhood of 6.0, 4.0, and 2.0 (*9*). The g anisotropy also gives rise to a pseudo-contact contribution to the shift observed, and a corresponding relaxation contribution. The shifts observed in Fig. 1B include the pseudo-contact contribution. Accurate estimates of the relaxation effects due to g anisotropy depend on a knowledge of orientational factors involving the g tensor and the cation–nucleus dipolar vector. However, these contributions are small compared to those computed above (Eq. [6]) by considering an isotropic g tensor (*12*).

Large ^{31}P contact shifts of ~2000 ppm (^{31}P NMR frequency of 108 MHz) were observed by Leroy and Guéron (*13*) in Co(II) complexes of AMP and ATP free in aqueous solutions. These experiments were performed with nucleotide concentrations in excess of 100 mM. Sternlicht *et al.* (*14*) estimated similar values for CoATP (^{31}P NMR frequency of 24 MHz) in earlier measurements on 350 mM samples. If similar shifts

were to occur in enzyme-bound complexes, ξ may become appreciable (10–30%). The motivation behind the current work was to ascertain if this is the case. A careful search was made for ^{31}P signals over a range of ± 2500 ppm and no signals other than those shown in Figs. 1A and 1B were found. The relatively small value of δ_C (<50 ppm) observed here is related to the contrasting nature of the sample conditions. At concentrations in excess of a few millimolar, free nucleotides stack in aqueous solutions and the complexes in the samples used in Refs. (*13*, *14*) are certain to be heavily stacked (*15*, *16*). Sigel *et al.* studied these effects exhaustively and have pointed out that structural conclusions concerning monomeric complexes based on measurements at high concentrations are generally rendered invalid due to stacking (*16*). The Co(II)–nucleotide complexes used in the present work, on the other hand, are exclusively in their enzyme-bound form, and are sequestered from other cation–nucleotide complexes. Stacking is, thus, not a factor in these enzyme–substrate complexes.

In conclusion, paramagnetic relaxation effects on nuclear relaxation in enzyme-bound Co(II) complexes of adenine nucleotides are primarily due to distance dependent cation–nucleus dipolar interactions, and are negligibly affected by contact interactions.

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