Field-Dependent Aluminum-27 NMR Studies of the Transferrins: An Approach for the Study of Metal Ion Binding Sites in Larger Proteins

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Field-Dependent Aluminum-27 NMR Studies of the Transferrins: An Approach for the Study of Metal Ion Binding Sites in Larger Proteins

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Abstract: The aluminum/carbonate derivatives of three transferrins—ovotransferrin and its half-molecules, serotransferrin and lactoferrin—have been studied by ^{27}Al NMR spectroscopy at four magnetic fields. In the case of ovotransferrin, one observes two ^{27}Al signals, corresponding to Al^{3+} bound to both metal ion-binding sites in the protein. However, for both serotransferrin and lactoferrin, these signals are degenerate at every field used in this study. In each case, only the central ($m = {}^1/_2 \rightarrow -{}^1/_2$) quadrupolar transition is observed; its detection is facilitated by the high molecular weights of these proteins. Moreover, for each transferrin-bound ^{27}Al signal, increasing the magnetic field leads to a downfield shift in peak position (termed the second-order dynamic frequency shift) and a decrease in line width. From the field dependence of the chemical shift, we have obtained values for the quadrupole coupling constant (χ) of Al^{3+} bound to the metal ion-binding sites of each protein ranging from $\chi = 3.3$ to 4.1 MHz. The values of χ for the half-molecules of ovotransferrin and the corresponding sites in the intact protein are virtually identical. Using these χ values and the field dependence of the line width, we calculated the rotational correlation time (τ_c) of bound Al^{3+} in each protein. For the intact transferrins, τ_c ranged from ≈40 to 60 ns, while significantly shorter values of τ_c were found for the half-molecules of ovotransferrin ($\tau_c \approx$ 14–17 ns). This report constitutes the first complete field-dependent investigation of a quadrupolar nucleus in isotropic motion under far from extreme narrowing conditions and demonstrates how quadrupolar metal ion NMR may be used to obtain physical information about the nature of the metal-binding sites of larger proteins.

Introduction

The use of nuclear magnetic resonance (NMR)[1] spectroscopy for the study of proteins has gained appreciable momentum over the last decade. The introduction of homonuclear two-dimensional and, subsequently, heteronuclear three- and four-dimensional NMR experiments has facilitated the elucidation of the solution structures of several small proteins.[2] These studies all rely on the detection of nuclei with nuclear spin I equal to $^1/_2$. However, quadrupolar nuclei—those with spin greater than $^1/_2$—comprise the vast majority of NMR active nuclei. In spite of this, quadrupolar NMR spectroscopy has been for the most part overlooked as a useful spectroscopic tool, especially for the study of biologically important molecules.[3] This is largely due to the fact that signals for quadrupolar nuclei are generally somewhat broader than those for $I = {}^1/_2$ nuclei. In addition, it is commonly assumed that the line width of the quadrupolar resonance will increase proportionally with molecular weight, as is the case for spin $^1/_2$ nuclei, leading to broad, unobservable signals. Hence, up to now, quadrupolar NMR has only been applied to the study of small metal ion-binding proteins (i.e., ^{43}Ca NMR) and to the weak (fast exchange) binding of ions to biological molecules.[4,5] However, recent ^{51}V and ^{27}Al NMR studies concerned with the binding of VO_2^+ and Al^{3+} to serotransferrin and ovotransferrin have demonstrated that it is possible to detect relatively narrow signals for quadrupolar metal ions that are tightly bound to the metal-binding sites of large metalloproteins.[6-8]

In this paper we present a field-dependent study of the ^{27}Al NMR signals of bound Al^{3+} in transferrins, a class of large (MW ≈ 80 000) Fe^{3+}-binding proteins.[9] Transferrins consist of two homologous lobes, each containing one high-affinity Fe^{3+}-binding site. Transferrins may also strongly bind a host of other metal ions, including Al^{3+}. An intriguing property of these proteins is the requirement of an anion (*in vivo*, carbonate) to facilitate metal ion binding in each site. We will show that field-dependent quadrupolar NMR provides a novel method of gaining insight into the symmetry of metal ion-binding sites and the motions of bound metal ions in proteins. In addition, these results also indicate that it should be feasible under appropriate conditions to obtain interpretable NMR signals for certain quadrupolar nuclei in large macromolecules.

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(1) Abbreviations used: NMR, nuclear magnetic resonance; OTf, ovotransferrin; sTf, serotransferrin; lTf, lactoferrin; OTf/2N, N(amino)-terminal half-molecule of ovotransferrin; OTf/2C, C(carboxy)-terminal half-molecule of ovotransferrin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TMS, tetramethylsilane; NOE, nuclear Overhauser enhancement.

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Experimental Section

Materials. The apo-forms of chicken OTf, human sTf, and human lTf were purchased from Sigma Chemical Co. and used without further purification. The N- and C-terminal half-molecules of OTf were produced from the intact protein by mild proteolysis with TPCK-treated trypsin according to published methods.[7,10] Aluminum nitrate, potassium chloride, tris(2,4-pentanedionato)aluminum ($Al(acac)_3$), and benzene-d_6 were obtained from Aldrich Chemical Co. Deuterium oxide and ^{13}C-labeled (99%) sodium carbonate were purchased from Cambridge Isotope Laboratories and MSD Isotopes, respectively.

NMR Spectroscopy. Protein NMR samples were prepared as previously described.[7] Protein concentrations were determined either spectrophotometrically (ϵ_{280} (M^{-1} cm^{-1}), for apo-OTf, 91 200; apo-sTf, 92 300; apo-lTf, 89 600) or, in the case of OTf/2N and OTf/2C, by weight. ^{27}Al NMR spectra were acquired at 25 °C on four instruments: Bruker ARX 300 (ν_0 = 78.2 MHz), AM 400 (ν_0 = 104.3 MHz), AMX 500 (ν_0 = 130.3 MHz), and AMX 600 (ν_0 = 156.4 MHz), each equipped with a 10-mm broad-band probe. Data were acquired using parameters given elsewhere,[7] zero-filled to $8K$, and processed with a line broadening of 20–50 Hz. $^{13}C\{^1H\}$ and ^{27}Al NMR studies of a 250 mM solution of $Al(acac)_3$ in benzene-d_6 (degassed and sealed under argon) were performed at 25 °C on a Bruker AM 400 spectrometer. ^{13}C and ^{27}Al longitudinal relaxation times (T_1) for this complex were determined by the inversion–recovery method. All ^{27}Al NMR spectra are referenced to external 1.0 M $Al(NO_3)_3$ in D_2O. ^{13}C NMR signals for $Al(acac)_3$ are referenced to TMS.

Theory

In general, the dominant relaxation pathway for $I > {}^1/_2$ nuclei is the quadrupolar relaxation mechanism.[5,11,12] This process, like other nuclear relaxation mechanisms, is highly dependent on the nature of the molecule under investigation. For small molecules which tumble rapidly in solution, the product of the Larmor frequency of the nucleus, ω_0, and its correlation time, τ_c, is much less than 1. In this "extreme narrowing" limit, relaxation may be described by a single exponential decay. The longitudinal (T_1) and transverse (T_2) relaxation times characterizing this process are equivalent and are given by the following expression:[13]

$$\frac{1}{T_1} = \frac{1}{T_2} = \pi\Delta\nu_{1/2} = \frac{3\pi^2}{10}\frac{(2I+3)}{I^2(2I-1)}\chi^2\tau_c \qquad (1)$$

χ, the quadrupole coupling constant, is a parameter that provides a measure of the coupling between the nuclear quadrupole and the electric field gradient at the nucleus. χ reflects the symmetry of the local environment of the nucleus and can be determined using eq 1 only if τ_c is known.

For quadrupolar nuclei bound to very large molecules, such that $\omega_0\tau_c \gg 1$, relaxation is multiexponential. However, it has been predicted that in this limit of molecular motion, the central ($m = {}^1/_2 \rightarrow -{}^1/_2$) transition of a half-integer quadrupolar nucleus (i.e., $I = {}^3/_2, {}^5/_2, {}^7/_2$) can give rise to a relatively narrow signal, while the remaining transitions will broaden dramatically.[14–17] For $I = {}^5/_2$ nuclei, such as ^{27}Al, the line width of the signal for the central transition is[18]

$$\frac{1}{\pi T_2} = \Delta\nu_{1/2} = (4.9 \times 10^{-3})\left(\frac{\chi^2}{\nu_0^2\tau_c}\right) \qquad (2)$$

Notice that the line width of this resonance actually *decreases* with increasing τ_c and that it is dependent on the resonance frequency of the nucleus, ν_0, which in turn is dependent on the external magnetic field, B_0. These trends are in sharp contrast to the situation described above for $\omega_0\tau_c \ll 1$ and to other nuclear relaxation processes, such as dipolar relaxation, where the line width increases with increasing $\omega_0\tau_c$.[19] In addition, the signal due to the central transition is thought to be shifted to lower frequency (i.e., upfield); a phenomenon termed the "second-order dynamic frequency shift".[16] The magnitude of this shift, which is 0 when $\omega_0\tau_c \ll 1$, decreases with increasing field and for $I = {}^5/_2$ nuclei is[20]

$$\Delta\delta_d = -(6 \times 10^3)\left(\frac{\chi^2}{\nu_0^2}\right) \qquad (3)$$

Expressions similar to eqs 2 and 3 may also be derived for $I = {}^3/_2$ and ${}^7/_2$ nuclei. Thus, from the field dependence of the chemical shift and line width of the signal due to the central transition, one can, in principle, obtain values of χ and τ_c for a quadrupolar nucleus under far from extreme narrowing conditions.

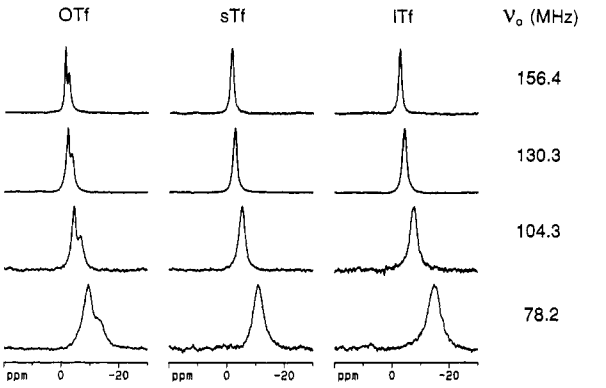

Figure 1. ^{27}Al NMR spectra of OTf (1.13 mM, pH 7.5), sTf (1.09 mM, pH 7.3), and lTf (0.73 mM, pH 7.5) at four magnetic fields: 7.0 T (ν_0 = 78.2 MHz), 9.4 T (ν_0 = 104.3 MHz), 11.7 T (ν_0 = 130.3 MHz), and 14.1 T (ν_0 = 156.4 MHz); (1 – 2) $\times 10^5$ scans each.

Results

^{27}Al NMR Studies of the Transferrins. ^{27}Al NMR spectra of the Al^{3+}/CO_3^{2-} adducts of OTf, sTf, and lTf at four different magnetic fields are shown in Figure 1. Since transferrins are bilobal proteins, with each lobe containing one metal ion-binding site, one would expect to observe two ^{27}Al signals corresponding to Al^{3+} bound to the two sites; this is indeed the case for OTf.[7,8] In contrast, ^{27}Al signals due to bound Al^{3+} in the two sites of sTf and lTf could not be resolved at any field used in this study. Spectra for the N- and C-terminal half-molecules of OTf were also obtained (Figure 2). As noted previously,[7,8] the ^{27}Al (and ^{13}C) resonances of the intact protein line up perfectly with those in the half-molecules, thus allowing their assignment. However,

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(13) $\chi = e^2qQ/h$, where e is the charge on the electron, q is the electric field gradient at the nucleus, Q is the nuclear quadrupole moment, and h is the Planck constant. The asymmetry term, $1 + \eta^2/3$, has been ommitted from eq 1.
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(18) Equation 2 was derived from the transverse relaxation matrix for $I = {}^5/_2$ nuclei in the slow motion limit: R_2 ($m = {}^1/_2 \rightarrow -{}^1/_2$) = $[8J(\omega) + (28 - 6\sqrt{10})J(2\omega)]K$, where $K = (1/200)(eQ/\hbar)^2$, and $J(\omega) = [3(eq)^2]/(10\omega^2\tau_c)$. Higher-order contributions to R_2 ($m = {}^1/_2 \rightarrow -{}^1/_2$) have been neglected; see ref 17.
(19) Jardetzky, O.; Roberts, G. C. K. *NMR in Molecular Biology*; Academic Press: New York, 1981; pp 44–68.
(20) The dynamic frequency shift is normally given in terms of angular frequency: $\Delta\omega_d = -(6 \times 10^{-3})(\chi^2/\omega_0)$, where $\chi = e^2qQ/\hbar$. By making the following subtitutions, $\Delta\delta_d = (\Delta\omega_d/\omega_0) \times 10^6$, $\omega_0 = 2\pi\nu_0$, and $\hbar = h/2\pi$, one obtains eq 3, in which $\chi = e^2qQ/h$.

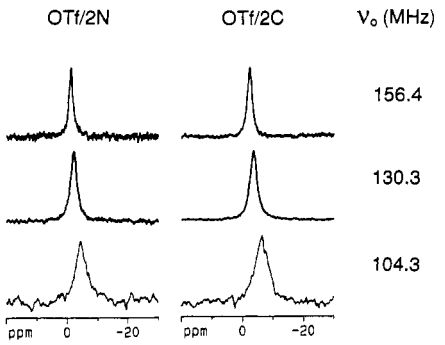

Figure 2. ^{27}Al NMR spectra of OTf/2N (0.19 mM, pH 7.6) and OTf/2C (0.30 mM, pH 7.7) at three magnetic fields: 9.4 T (ν_0 = 104.3 MHz), 11.7 T (ν_0 = 130.3 MHz), and 14.1 T (ν_0 = 156.4 MHz); (1 – 1.3) ×10^6 scans each.

at any given field, the line widths of the ^{27}Al signals for Al^{3+} bound to the half-molecules are much larger than the corresponding signals in the intact protein (see Table I). The spectra in Figures 1 and 2 clearly show that as the magnetic field is increased, each transferrin-bound ^{27}Al signal becomes narrower and moves to higher frequency (i.e., downfield). These trends are in agreement with eqs 2 and 3 and are among several that are characteristic of the $m = {}^1/_2 \rightarrow -{}^1/_2$ transition of $I = {}^n/_2$ nuclei (n = 3, 5, 7) in the limit of slow molecular motion.[6-8] Plots of the chemical shift and line width of each protein-bound ^{27}Al signal against the inverse of the square of the nuclear resonance frequency are shown in Figures 3 and 4. For each resonance, the field dependence of the chemical shift and the line width can be fit to a straight line ($r^2 \geq 0.98$). A value for χ can be calculated from the slope of each line in Figure 3 using eq 3. The y-intercepts of the lines in Figure 3 give the unshifted ("isotropic") chemical shifts (δ_i) of the bound ^{27}Al signals in these proteins ($\delta_i \approx$ 1–2 ppm), which fall well within the chemical shift window diagnostic of octahedral Al^{3+}.[21] With these χ values in hand, the correlation time of the bound metal ion in each site can then be computed from the field dependence of the line width of each signal (Figure 4) using eq 2. A complete list of the ^{27}Al NMR data obtained in this fashion for each protein is shown in Table I.

^{13}C and ^{27}Al NMR Studies of Al(acac)$_3$. By way of a comparison, we have also obtained values of χ and τ_c for a model aluminum complex, tris(2,4-pentanedionato)aluminum (Al(acac)$_3$). In this complex, six oxygen atoms form a near perfect octahedral ligand coordination sphere about the central metal ion.[22] For a molecule of this size, extreme narrowing conditions apply. Hence, upon determination of the τ_c of Al(acac)$_3$, the quadrupole coupling constant of Al^{3+} in this complex could be calculated from the T_1 or T_2 of its ^{27}Al NMR signal using eq 1. The correlation time of Al(acac)$_3$ was obtained from the T_1 relaxation time of the ^{13}C signal due to the three C–H groups in the complex. From NOE experiments, these carbons relax exclusively by dipolar relaxation (i.e., $\eta \approx 2.0$), and the τ_c of the molecule ($\tau_c = 4.2 \times 10^{-11}$ s) can then be calculated using the following expression:[23]

$$\frac{1}{T_1^{DD}} = \left(\frac{\mu_0}{4\pi}\right)^2 \left(\frac{N_H \gamma_H{}^2 \gamma_C{}^2 \hbar^2 \tau_c}{r_{CH}{}^6}\right) \qquad (4)$$

From the ^{27}Al NMR data for this complex, a value of χ for the central metal ion was calculated from eq 1 (χ = 3.1 MHz). Our experimental data for the Al(acac)$_3$ complex, shown in Table II, are in good agreement with previous work,[24] except that these authors underestimated χ by a factor of 2π (J. Kowalewski, private communication).

Discussion

In this paper we have used the field dependence of the chemical shift and line width of the central transition of a quadrupolar nucleus (^{27}Al) to determine the quadrupole coupling constant and rotational correlation time of a quadrupolar metal ion bound to a large protein. To the best of our knowledge, this is the first field-dependent study of a quadrupolar nucleus in isotropic motion under far from extreme narrowing conditions, though this methodology has been known to be applicable to the extraction of quadrupole coupling constants for quadrupolar nuclei in solids for some time.[25]

The values of χ for Al^{3+} bound to the iron-binding sites in the transferrins range from χ = 3.3 to 4.1 MHz and are quite comparable to that for Al^{3+} in Al(acac)$_3$ (χ = 3.1 MHz). To date, literature values of χ for bound Al^{3+} in a variety of complexes determined by NMR and nuclear quadrupole resonance (NQR) range from $1 < \chi > 20$;[26] this suggests a relatively high degree of symmetry for the metal ion in the sites of each protein. This notion is supported by recent X-ray crystallographic studies of the Fe^{3+} forms of intact lTf[27] and the N-terminal lobe of rabbit sTf,[28] in which the metal ion in each site is surrounded by a slightly distorted octahedral arrangement of ligands (1 Asp, 1 His, and 2 Tyr, plus two oxygens from carbonate). In addition, these results indicate that the symmetry of metal ion sites in these proteins decrease in the order

$$\text{OTf N} > \text{sTf N, C} > \text{OTf C} > \text{lTf N, C}$$

Note that the values of χ for Al^{3+} in the half-molecules of OTf and the corresponding sites in the intact protein are virtually identical, suggesting that proteolysis of the loop that connects the two lobes of the protein does not alter the physical integrity of the metal ion-binding sites in the isolated halves.

The correlation times for Al^{3+} in intact transferrins ($\tau_c \approx$ 40–60 ns) are significantly longer than those for the half-molecules of OTf ($\tau_c \approx$ 14–17 ns).[29] Our results are in good agreement with a recent published value for the τ_c of OTf (≈33 ns) obtained by perturbed angular correlation (PAC) techniques.[30,31] Furthermore, these authors suggest that the correlation times of the half-molecules of OTf are somewhat shorter. In addition, the τ_c values obtained for the proteins in this study are comparable to the correlation times of other globular proteins of similar molecular weight determined by fluorescence and NMR,[32] suggesting that the bound Al^{3+} ions do not move with respect to the protein. Our

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(29) Note that for each protein, the value of $\omega_0\tau_c$ is much greater than 1 for ^{27}Al at every field used in this study, allowing us to employ eqs 2 and 3 with confidence.
(30) Schwab, F. J.; Appel, H.; Neu, M.; Thies, W.-G. *Eur. Biophys. J.* **1992**, *21*, 147–154.
(31) The correlation times for Al^{3+} bound to OTf, sTf, and lTf are somewhat different than the values predicted some time ago using the Stokes–Einstein equation (Koenig, S. H.; Schillinger, W. E. *J. Biol. Chem.* **1969**, *244*, 3283–3289. Harris, D. C.; Gray, G. A.; Aisen, P. *J. Biol. Chem.* **1974**, *249*, 5261–5264).

Table I. ^{27}Al NMR Data for the Al^{3+}/Carbonate Derivatives of the Transferrins

		B_0 = 14.1 T (ν_0 = 156.4 MHz)		B_0 = 11.7 T (ν_0 = 130.3 MHz)		B_0 = 9.4 T (ν_0 = 104.3 MHz)		B_0 = 7.0 T[a] (ν_0 = 78.2 MHz)			
protein	site	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	χ (MHz)	τ_c (ns)
OTf	N	−1.2	100	−2.3	140	−4.6	180	−9.4	260	3.3	43
	C	−2.4	150	−3.8	170	−6.8	240	−13.2	320	3.8	48
sTf	N, C	−1.6	170	−2.8	180	−5.4	230	−11.1	300	3.6	58
lTf	N, C	−2.7	160	−4.3	200	−7.7	280	−15.1	390	4.1	44
OTf/2N		−1.2	220	−2.3	270	−4.6	410	n.d.	n.d.	3.3	14
OTf/2C		−2.3	260	−3.7	300	−6.5	460	n.d.	n.d.	3.7	17

[a] ^{27}Al signals for OTf/2N and OTf/2C were observed at this field, but because of the large line widths, only spectra with poor signal/noise ratios could be obtained; hence, no reliable values for δ and $\Delta\nu_{1/2}$ could be measured (n.d.).

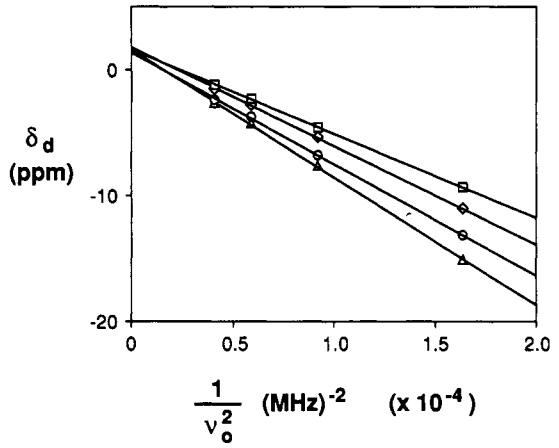

Figure 3. Field dependence of the chemical shifts of transferrin-bound ^{27}Al signals. (□) OTf N-site; (○) OTf C-site; (◇) sTf; (△) lTf. To avoid confusion, the data for OTf/2N and OTf/2C have not been included in this plot. The chemical shift data (and thus the χ values) for the half-molecules are virtually identical to those for the corresponding sites in intact OTf (see Table I).

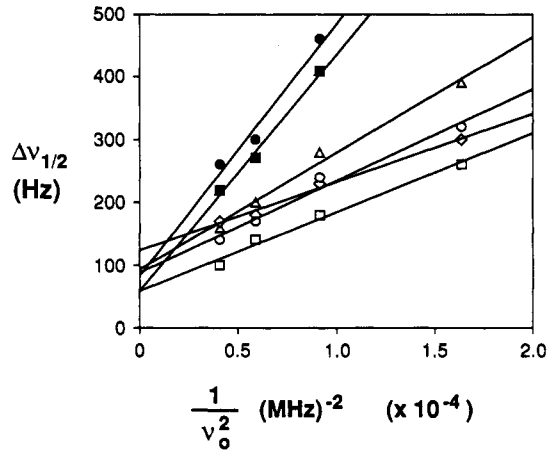

Figure 4. Field dependence of the line widths of transferrin-bound ^{27}Al signals. (□) OTf N-site; (○) OTf C-site; (◇) sTf; (△) lTf; (■) OTf/2N; (●) OTf/2C.

τ_c data also account for the much larger line widths of the ^{27}Al signals for the half-molecules compared to native OTf, as predicted by quadrupolar relaxation theory (eq 2) and in agreement with ^{13}C NMR data for these proteins.[8] Moreover, these results indicate that the motion of the lobes in the intact protein is restricted. This is quite different from other bilobal proteins, such as calmodulin, where the correlation times of the isolated half-molecules and the native protein are virtually identical, suggesting that in calmodulin the lobes can move independent from each other.[33]

Table II. ^{13}C and ^{27}Al NMR Data for $Al(acac)_3$

a. ^{13}C NMR[a]

δ (ppm)	η	T_1^{DD} (s)	τ_c (s)
101.1	2.0	1.1	4.2×10^{-11}

b. ^{27}Al NMR

δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)	T_2 (ms)	χ (MHz)
−0.9	2.6	123	2.6	3.1

[a] The ^{13}C data shown are for the signal due to the methine group in the ligand.

In conclusion, this field-dependent ^{27}Al NMR study of the transferrins demonstrates that quadrupolar NMR can provide a unique means of shedding light on the nature of the metal ion-binding sites in large metalloproteins. Since the majority of NMR nuclei in the periodic table share most of the quadrupolar properties of ^{27}Al, similar approaches may be suitable for a broad spectrum of biological applications. One can easily envisage experiments involving other tightly bound quadrupolar metal ions or anions and quadrupolar probes incorporated into large proteins using recombinant techniques. It is likely that this technique will be most readily applicable to those quadrupolar nuclei with small quadrupole moments (Q) and high resonance frequencies (ν), provided that the environment of the bound nucleus in the protein is relatively symmetric (i.e., low χ). Interestingly, because of the peculiar relaxation behavior of the quadrupole central transition, the best results at any given magnetic field are obtained when using larger proteins, lower temperatures, and higher viscosity;[34] these conditions are all opposite to what one normally uses for spin $^1/_2$ nuclei which relax by other mechanisms. In addition, the detectability of the central transition is even more dramatically enhanced at higher magnetic fields compared to spin $^1/_2$ nuclei. Thus, access to modern high field NMR spectrometers (i.e., 750 MHz or higher) would greatly facilitate future quadrupolar NMR studies of large biomolecules.

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