Quadrupolar Metal Ion NMR Study of Ovotransferrin at 17.6 T

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The increased sensitivity and resolution of high-field nuclear magnetic resonance (NMR) spectrometers, combined with the burgeoning arsenal of multidimensional techniques for the biologically relevant spin $I = {}^1/_2$ nuclei (i.e., ^{1}H, ^{13}C, ^{15}N, and ^{31}P), has made NMR spectroscopy the most powerful technique for probing the solution structures and dynamics of biological macromolecules.[1] The now commercially available 750-MHz (17.6 T) instrument was manufactured with these objectives in mind. However, recent ^{27}Al, ^{45}Sc, and ^{51}V NMR studies[2-5] of the transferrins, a class of MW ≈ 80 000, non-heme, Fe^{3+}-binding proteins,[6] have established what we believe could become a second major application of high-field NMR instruments, namely, the study of quadrupolar ($I > {}^1/_2$) nuclei bound to large biomolecules. In this report, we have investigated three trivalent metal ions (Al^{3+}, Sc^{3+}, and Ga^{3+}) bound to chicken ovotransferrin (OTf) by ^{27}Al ($I = {}^5/_2$), ^{45}Sc ($I = {}^7/_2$), and ^{71}Ga ($I = {}^3/_2$) NMR at a magnetic field of 17.6 T. Aside from containing some of the first 750-MHz data of any kind, this study will demonstrate the dramatic improvements in the detectability of such nuclei at this field compared to our previous work.

For half-integer quadrupolar nuclei (i.e., $I = {}^n/_2$, $n = 3, 5, 7$) in the limit of slow isotropic molecular motion, as is the case for moderate- to high-frequency nuclei bound to large proteins (i.e., $\omega_0\tau_c \gg 1$), quadrupolar relaxation theory predicts that the central ($m = {}^1/_2 \rightarrow -{}^1/_2$) transition can give rise to a relatively narrow signal whose line width, $\Delta\nu_{1/2}$, decreases with increasing magnetic field, $\mathbf{B}_0$, according to eq 1:[7-10]

$$\Delta\nu_{1/2} = k\left(\frac{\chi^2}{\nu_0^2\tau_c}\right) \qquad \begin{aligned} I &= {}^3/_2, \quad k = 2.0 \times 10^{-2} \\ I &= {}^5/_2, \quad k = 4.9 \times 10^{-3} \\ I &= {}^7/_2, \quad k = 2.5 \times 10^{-3} \end{aligned} \tag{1}$$

where χ, the quadrupole coupling constant, is a measure of the symmetry of the electronic environment of the nucleus,[11] τ_c is the correlation time of the fluctuations in the electric field gradient at the nucleus, and ν_0 is its resonance frequency (which is proportional to $\mathbf{B}_0$). The chemical shift of this resonance is also highly dependent on the strength of the magnetic field, and this so-called second-order dynamic frequency shift" is[10,12,13]

$$\Delta\delta_d = -k\left(\frac{\chi^2}{\nu_0^2}\right) \qquad \begin{aligned} I &= {}^3/_2, \quad k = 2.5 \times 10^4 \\ I &= {}^5/_2, \quad k = 6.0 \times 10^3 \\ I &= {}^7/_2, \quad k = 2.5 \times 10^3 \end{aligned} \tag{2}$$

We have shown that physical information (i.e., χ and τ_c) for a quadrupolar nucleus in the slow motion limit may be obtained from the field dependence of the line width and the chemical shift of the signal due to the central transition using the above expressions.[2a,3,4]

^{27}Al, ^{45}Sc, and ^{71}Ga NMR spectra of the M^{3+}/CO_3^{2-} forms of OTf (M = Al, Sc, and Ga) at a magnetic field of 17.6 T are presented in Figure 1.[14] When a saturating amount of metal ion is added to OTf, as in the ^{27}Al and ^{45}Sc spectra shown, signals due to the metal ion bound in slow exchange to both sites of the bilobal protein are observed; these resonances have been assigned using the proteolytic half-molecules of OTf (OTf/2N and OTf/2C).[2-4,15] In the third case, we have found that OTf shows a marked preference for binding Ga^{3+} at its N-site[16] (analogous to experiments where Al^{3+} is the titrant[2a]); hence, only one ^{71}Ga signal can be detected when a subequimolar amount of Ga^{3+} is added to this protein. In each spectrum, the estimated isotropic chemical shift (δ_i) for each signal (i.e., the resonance position in the absence of a dynamic frequency shift), deduced from plots of the chemical shift *vs* the inverse of the square of the nuclear resonance frequency, is represented with a dashed line. These values are listed in Table 1 along with the data for the adducts from this study and those recorded at a field of 11.7 T.[3,4,16]

For all three metal nuclei, the line widths of the protein-bound signals drop significantly at a $\mathbf{B}_0$ of 17.6 T compared to 11.7 T, resulting in a substantial improvement in both the detectability and the resolution of such signals (see Table 1). Notice that this enables one to obtain excellent spectra with the use of less sample and an inherently less sensitive assembly (i.e., we used a 5-mm inverse probe in this study compared to standard 10-mm probes

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(6) The three main types of transferrins—serotransferrin, ovotransferrin, and lactoferrin—are highly homologous bilobal proteins. Each lobe contains one high-affinity Fe^{3+}-binding site, and metal ion binding can occur only if an anion (*in vivo*, carbonate) is present. Recent X-ray structures of human lactoferrin (Anderson, B. F.; Baker, H. M.; Norris, G. E.; Rice, D. W.; Baker, E. N. *J. Mol. Biol.* **1989**, *209*, 711–734) and the N-terminal lobes of rabbit serotransferrin (Sarra, R.; Garratt, R.; Gorinsky, B.; Jhoti, H.; Lindley, P. *Acta Crystallogr.* **1990**, *B46*, 763–771) and ovotransferrin (Dewan, J. C.; Mikami, B.; Hirose, M.; Sacchettini, J. C. *Biochemistry* **1993**, *32*, 11963–11968) have revealed that the metal ion is bound to the side chains of four highly conserved protein residues (one Asp, one His, and two Tyr) plus carbonate (bidentate) in a slightly distorted octahedral geometry.
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(11) The quadrupole coupling constant is commonly given by the expression $\chi = e^2qQ/h$, where *eq* is the electric field gradient at the nucleus and *eQ* is the nuclear quadrupole moment. Quadrupole moments for the nuclei used in this study are as follows: Q (10^{-28} m^2) ^{27}Al, 0.14; ^{45}Sc, –0.22; ^{71}Ga, 0.11 (Mills, I.; Cvitas, T.; Homann, K.; Kallay, N.; Kuchitsu, K. *Quantities, Units and Symbols in Physical Chemistry*, 2nd ed.; Blackwell Scientific Publications: Oxford, 1993; pp 98–104). In addition, two assumptions are implicit in both eq 1 and eq 2: molecular motion is isotropic, and the field gradient at the nucleus is cylindrically symmetric.
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(14) The suppliers of chemicals used in this work in addition to the procedures for the preparation and titration of protein NMR samples are described in our earlier reports (ref 2a, 3, 4, herein). ^{27}Al (ν_0 = 195.5 MHz), ^{45}Sc (ν_0 = 182.2 MHz), and ^{71}Ga (ν_0 = 228.8 MHz) NMR spectra were acquired locked at 25 °C on a Bruker AMX-2 750 spectrometer equipped with a 5-mm broad band inverse probe using the following parameters: a 20–30° flip angle, repetition times of 42, 18, and 5 ms, sweep widths of 50–125 kHz, 1–4 K data points, and dead times of 240, 22, and 30 μs for ^{27}Al, ^{45}Sc, and ^{71}Ga, respectively. Data were zero-filled to 4 K and processed with line broadening factors of 20, 100, and 250 Hz, respectively. The LINESIM program (P. Barron, Bruker Australia) was used to obtain chemical shifts and line widths of overlapping signals (i.e., in the ^{27}Al and ^{45}Sc spectra). ^{27}Al, ^{45}Sc, and ^{71}Ga NMR spectra are referenced to external 1.0 M $Al(NO_3)_3/D_2O$, 1.0 M $ScCl_3/D_2O$, and 1.0 M $Ga(NO_3)_3/D_2O$, respectively.
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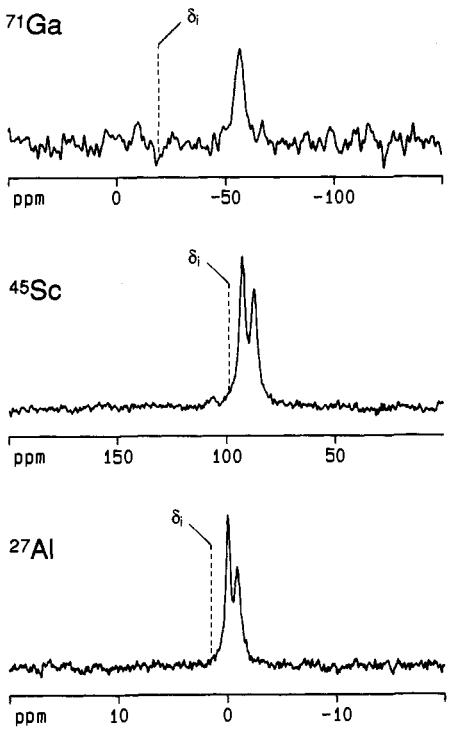

Figure 1. ^{27}Al, ^{45}Sc, and ^{71}Ga NMR spectra (B_0 = 17.6 T) of the Al^{3+}, Sc^{3+}, and $Ga^{3+}/^{13}CO_3^{2-}$ forms of OTf (1.02 mM, 20 mM $Na_2{}^{13}CO_3$, 0.1 M KCl, 25% v/v D_2O). ^{27}Al: 2.0 equiv of Al^{3+}, pH 7.5, 22 600 scans, 16 min. ^{45}Sc: 1.8 equiv of Sc^{3+}, pH 7.8, 400 000 scans, 2 h. ^{71}Ga: 0.8 equiv of Ga^{3+}, pH 7.7, 600 000 scans, 50 min. The extrapolated isotropic chemical shift (δ_i) of the resonances in each spectrum is denoted by a dashed line; in the ^{27}Al and ^{45}Sc spectra, the average δ_i of the two signals is shown.

Table 1. ^{27}Al, ^{45}Sc, and ^{71}Ga NMR Data for the M^{3+}/Carbonate Derivatives of OTf at 17.6 and 11.7 T (M = Al, Sc, Ga)

		B_0 = 17.6 T		B_0 = 11.7 T[b]		
nucleus	site[a]	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	δ_i[c] (ppm)
^{27}Al	N	−0.1	80	−2.3	140	1.6
	C	−0.9	115	−3.8	170	1.4
^{45}Sc	N	93	490	85	740	99
	C	87	540	77	940	98
^{71}Ga	N	−57	860	−103	1900	−19

[a] Assignments were determined by studies of the tryptic N- and C-terminal half-molecules of OTf (OTf/2N and OTf/2C). [b] These data were obtained from refs 3, 4, and 16. [c] The isotropic chemical shifts (δ_i) for each signal were determined from plots of the field dependence of their chemical shift (see refs 3, 4). For the ^{71}Ga signal due to the N-site of OTf, the chemical shift at a third field (δ = −151 ppm, B_0 = 9.4 T) was also used in the calculation of δ_i (ref 16).

in our earlier work[2-4]). In addition, the chemical shift of each signal from the 750-MHz instrument is downfield of the same resonance detected on the lower field spectrometer. Both of the above effects are in agreement with eqs 1 and 2. The appreciable changes in both line width and chemical shift observed for the OTf-bound ^{71}Ga resonance compared to the results for the other nuclei can be largely attributed to the fact that the leading coefficients in these equations decrease with increasing nuclear spin. Using the data reported here and our earlier results,[3,4] we can predict that a further increase in the external magnetic field, B_0 (i.e., say to 23.4 T on a putative 1-GHz instrument), would result in only minor changes in the ^{27}Al and ^{45}Sc spectra of OTf. However, under these conditions, both the chemical shift and the line width of the ^{71}Ga resonance would still be significantly altered. Note that the virtually identical ^{27}Al, ^{45}Sc, and ^{71}Ga isotropic chemical shifts due to the bound metal ion in these sites indicate that one will eventually reach a field where the signals are virtually degenerate. Another point to keep in mind is that at extremely high fields one may reach a stage where contributions to the observed signal line width by other nuclear relaxation mechanisms, especially CSA, which increases with the square of the field strength,[17] cannot be flippantly neglected. There are examples in the literature which prove that CSA relaxation can be a very real component of the relaxation of a quadrupolar nucleus under nonextreme narrowing conditions.[18] Of course, this should not perturb the second-order dynamic frequency shift (and hence the determination of χ), which is a purely quadrupolar effect. Moreover, the excellent linear fits of the line width data for each nucleus ($r^2 \geq 0.98$) suggest that under these conditions quadrupolar relaxation remains the dominant pathway in all of our experiments (graphs not shown).

Based on the results presented in this article, we feel that the true value of high-field quadrupolar NMR for the study of macromolecules will be realized for those nuclei which are difficult or impossible to detect on lower field instruments due to factors such as larger Q and q terms (i.e., χ) and lower values of ν_0 and τ_c (though still in the $\omega_0\tau_c \gg 1$ limit). For example, from eq 1, one can calculate that at any field the OTf-bound signal for the second NMR-active isotope of gallium, ^{69}Ga ($I = {}^3/_2$), whose quadrupole moment, Q, is larger than that of ^{71}Ga (0.17 *vs* 0.11 b)[11] and whose resonance frequency is a factor of 1.27 lower,[19] should be ≈4 times broader than the ^{71}Ga resonance corresponding to the same site. By inspection of Table 1, we can postulate that the detection of OTf-bound ^{69}Ga signals will be feasible on a 750-MHz instrument, while such signals are almost broadened beyond detectability at a field strength of 11.7 T.[16] Moreover, while Al^{3+}, Sc^{3+}, and Ga^{3+} are useful surrogates for probing the binding of Fe^{3+} to transferrins, this methodology may be extended to other proteins containing biologically relevant quadrupolar nuclei, such as ^{25}Mg ($I = {}^5/_2$, Q = 0.20 b, ν_0 = 45.9 MHz at 17.6 T), ^{43}Ca ($I = {}^7/_2$, Q = −0.04 b, ν_0 = 50.5 MHz at 17.6 T), and ^{67}Zn ($I = {}^5/_2$, Q = 0.15 b, ν_0 = 46.9 MHz at 17.6 T).[11,19] Of these, ^{43}Ca is by far the best candidate because of its small quadrupole moment. Using an average literature χ value of 1 MHz for protein-bound ^{43}Ca[20] and a correlation time of 40 ns (i.e., transferrins[3,4]), eq 1 predicts that the line width of the central transition of this nucleus would be ≈25 Hz at 17.6 T; this is a far cry from the documented ^{43}Ca line widths for small proteins (≈200–800 Hz).[20] Quadrupolar central transition (QCT) NMR may, therefore, significantly expand the menu of NMR-active nuclei and their applications for the study of biologically important molecules.

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