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Soft-Pulsed Aluminum-27 Quadrupolar Central Transition NMR Studies of Ovotransferrin

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We have employed soft (Gaussian) pulses to examine ^{27}Al NMR signals arising from Al^{3+} bound to ovotransferrin. In addition to enhancing the general detectability of ^{27}Al signals from a practical standpoint, this technique makes it possible to invert the central component of the protein-bound ^{27}Al signals, providing a route for, to our knowledge, the first inversion-recovery measurements on such systems. The suitability of this approach for gaining insights into the nature of metal ion binding sites in large metalloproteins is critically assessed.

Key Words: **^{27}Al NMR; quadrupolar central transition; soft pulses; transferrin.**

Although quadrupolar nuclei, those with spin I greater than $\frac{1}{2}$, constitute the vast majority of NMR active nuclei in the periodic table, their unfavorable relaxation properties generally hamper their application to the study of large macromolecules in solution. However, a number of examples in the literature over the past decade, primarily involving high-affinity metal ion (^{27}Al (*1–5*), ^{45}Sc (*6*), ^{51}V (*7, 8*), $^{69,71}Ga$ (*9*)) binding to a family of large (MW $\approx$ 80,000) iron-binding and transport proteins, the transferrins, have opened the door to this area of study. This is made possible by the unique relaxation behavior of the central transition of half-integer quadrupolar nuclei, which in the limit of slow isotropic molecular motion (i.e., $\omega_0\tau_c \gg 1$) displays a number of traits that are unusual in solution-state NMR. These include a field-dependent chemical shift, the so-called second-order dynamic frequency shift

$$\delta_{obs} - \delta_{iso} \quad \alpha \quad \frac{\chi^2}{\nu_0^2}, \qquad [1]$$

and a signal linewidth that decreases with increasing magnetic field strength (*10–13*):

$$\Delta\nu_{1/2} \quad \alpha \quad \frac{\chi^2}{\nu_0^2\tau_c}. \qquad [2]$$

In several of the studies cited above it was shown that field-dependent changes to the chemical shift and linewidth can be used to extract information concerning the environment of the bound metal ion via two physical parameters, namely the quadrupole coupling constant, χ, and the rotational correlation time, τ_c.

In principle one could also use the longitudinal relaxation time, T_1, of the central transition in combination with the dynamic frequency shift as an alternative route to χ and τ_c. However, this approach has proven to be difficult because the use of hard radiofrequency pulses precludes the inversion of such signals (*1, 6–9*). This motivated us to explore the feasibility of employing soft (Gaussian) pulses in an attempt to invert, and subsequently obtain the T_1 of,

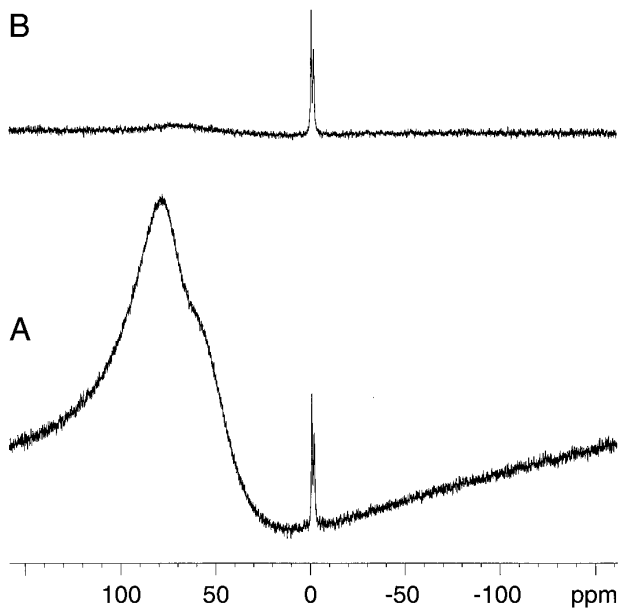

FIG. 1. ^{27}Al (156.4 MHz) NMR experiments of the Al^{3+}/carbonate adduct of 0.84 mM OTf at 283 K using (A) hard rectangular pulses (6 μs; RF pulse strength, ω_1 = 12.5 kHz); preacquisition delay = 140 μs; 1000 scans; and (B) Gaussian pulses (500 μs; RF pulse strength, ω_1 = 150 Hz); preacquisition delay = 14.3 μs; 5000 scans.

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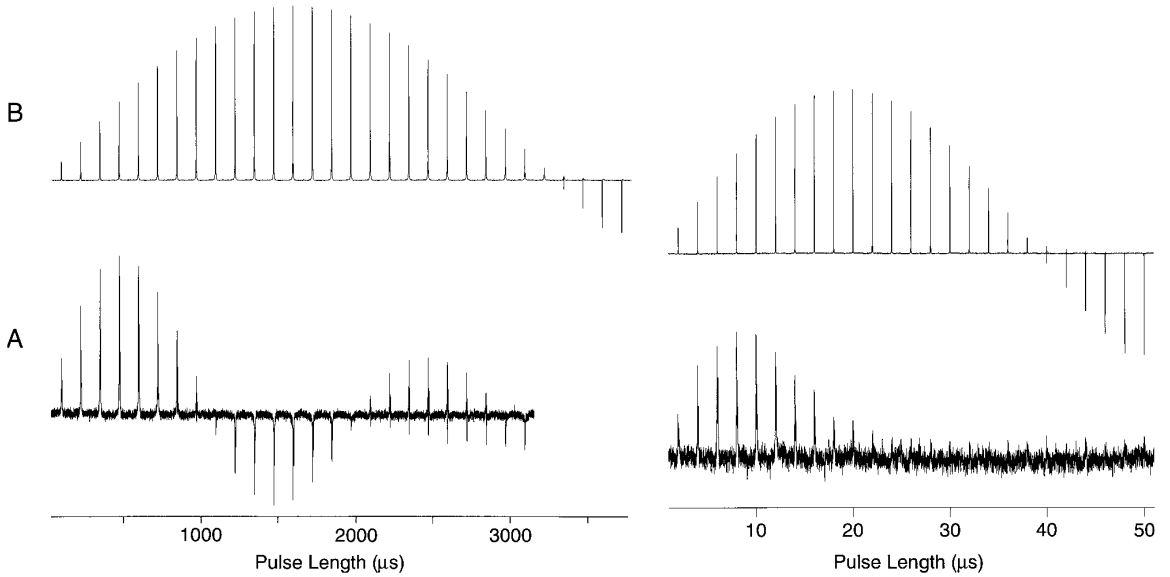

FIG. 2. (Left) Dependence of the ^{27}Al (156.4 MHz) NMR signals for solutions of (A) OTf-bound (283 K; 5000 scans each; 0.84 mM) and (B) free Al^{3+} (300 K; 1 scan each; 10 mM) on the length of the soft pulse (Gaussian pulses; ω_1 = 150 Hz). (Right) ^{27}Al pulse length dependence of OTf-bound (bottom; 256 scans each) and free Al^{3+} (top; 1 scan each) using hard rectangular pulses (ω_1 = 12.5 kHz).

^{27}Al signals due to Al^{3+} bound to chicken ovotransferrin (OTf). This metalloprotein, like the others in the transferrin class, is bilobal, with each lobe containing one high-affinity Fe^{3+}-binding site capable of strongly chelating a large array of metal ions; an anion, for example, carbonate, is absolutely required for tight metal ion binding (for a review, see (*14*)).

In Fig. 1 we show ^{27}Al QCT (quadrupolar central transition) NMR spectra (*15*) of the Al^{3+}/carbonate adduct of OTf obtained under identical conditions, with the exception of the radiofrequency strength. Using hard pulses, one excites a very wide frequency window, and consequently a very large undesired background signal due to aluminum present in the probehead materials is observed (Fig. 1A). To attenuate this huge signal, one must normally resort to an increased preacquisition delay (the time between the end of the radiofrequency pulse and the start of acquisition; 140 μs in the figure), which jeopardizes the detection of relatively broad signals of interest. However, soft pulses centered on the OTf-bound signals provide a very clean ^{27}Al spectrum that is free of the background signal even down to very low preacquisition delays (14.3 μs in Fig. 1B). Thus, soft pulses provide immediate dividends in the application of ^{27}Al NMR in cases where one may be dealing with broad signals.

Next, we investigated the pulse length dependence of the ^{27}Al-OTf signals when soft pulses are employed. Figure 2 compares a series of protein-bound and free Al^{3+} signals as a function of increasing Gaussian pulse length. We find that the protein-bound signals can indeed be inverted; as shown in the right panel of Fig. 2 this effect cannot be achieved using hard pulses. Moreover, as predicted for excitation of quadrupolar nuclei using a radiofrequency strength that is much less than the strength of the quadrupolar interaction

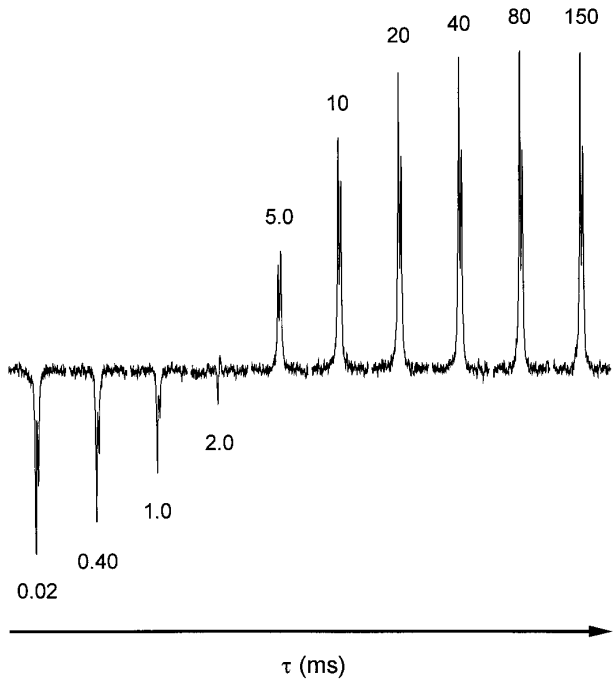

FIG. 3. Inversion-recovery (T_1) ^{27}Al (156.4 MHz) NMR spectra of the Al^{3+}/carbonate adduct of 0.84 mM OTf at 283 K acquired using Gaussian pulses (ω_1 = 150 Hz; 5000 scans each).

TABLE 1
^{27}Al Relaxation Data for OTf-Bound Al^{3+}

Site	T [K]	T_1 [ms][a]	T_2 [ms][b]	T_1/T_2
N	283	5.7	4.2	1.36
C	283	4.1	2.8	1.46
N	300	4.0	3.4	1.18
C	300	2.8	2.0	1.40

[a] T_1 relaxation times were obtained using the inversion-recovery technique and $\omega_1 = 150$ Hz.

[b] T_2 relaxation times were obtained from the signal linewidths, computed from the overlapping signals using our own least-squares fitting program (Kaleidograph 3.0, Macintosh), according to the expression $T_2 = (\pi \cdot \Delta\nu_{1/2})^{-1}$.

(i.e., $\omega_1 \lll \omega_Q$; the so-called ''fictitious spin $\frac{1}{2}$ formalism'' (*16–18*)), the detectable OTf-bound ^{27}Al signals, corresponding to the central transition, exhibit an intensity maximum at a pulse length, t_p, three times shorter than a 90° flip angle for a dilute solution of free metal ion:

$$t_p = \frac{\pi/2}{(I + \frac{1}{2})}. \qquad [3]$$

In this study, we found that the signal could be efficiently inverted only at radiofrequency strengths below 360 Hz, and that soft rectangular pulses could also be used (data not shown).

The ability to invert the protein-bound ^{27}Al signals through the use of soft pulses provides a means of performing inversion-recovery T_1 measurements (Fig. 3). We have obtained apparent T_1 values for both signals at 283 and 300 K, and find a T_1/T_2 ratio of ≈1.2 to 1.4 (Table 1). Note that there is a consistent difference in longitudinal relaxation between the two sites (i.e., T_1 N $> T_1$ C). In the limit of truly selective excitation of the central transition of an $I = \frac{5}{2}$ quadrupolar nucleus, the longitudinal relaxation for this component is predicted to be monoexponential, with an expression analogous to Eq. [2], and the T_1/T_2 ratio should be 0.898 ((*13*); C. Hughes and S. Wimperis, private communication). Hence, in our system the Gaussian pulses are not completely selective for the central component; this is demonstrated by the excitation profile shown in Fig. 4. Unlike the case of T_2 relaxation, perturbation of the outer components complicates the T_1 relaxation of the central component. Notice that, based on the published χ values for ^{27}Al bound to both sites of this protein, the outer components should resonate well within the Gaussian excitation window at the radiofrequency strength used in our experiments. Fur-

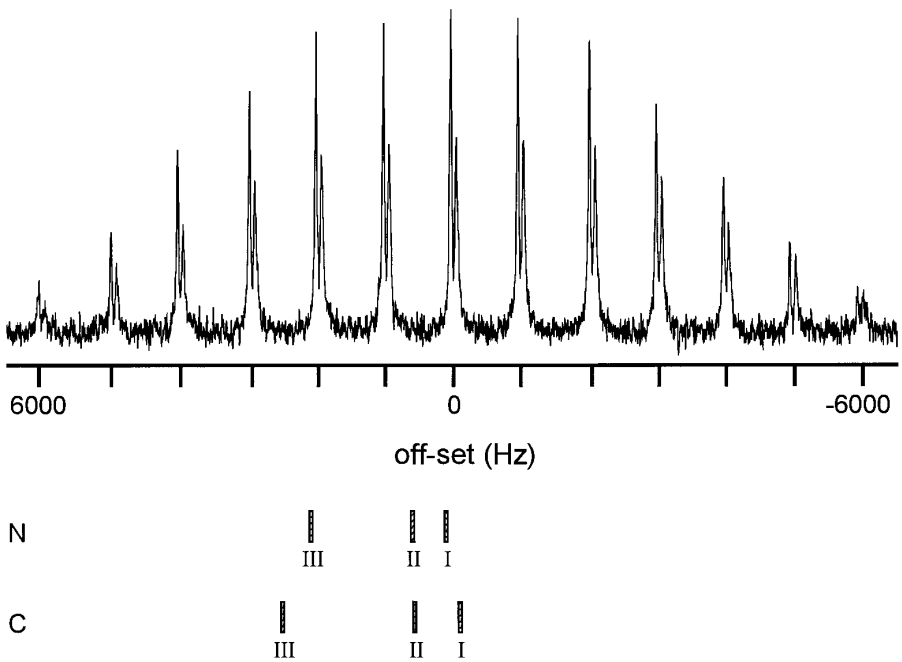

FIG. 4. Effect of frequency offset on the ^{27}Al (156.4 MHz) NMR spectra of the Al^{3+}/carbonate adduct of 0.84 mM OTf at 283 K obtained using Gaussian pulses ($\omega_1 = 150$ Hz; 2000 scans each). Below are the putative positions of the three components of the ^{27}Al signal for both sites, based on the respective χ (N, 3.4 MHz; C, 3.9 MHz) and δ_{iso} (N, +1.6 ppm; C, +1.4 ppm) values that we have reported (*2, 3*), and expressions for each component of the form of Eq. [1] (i.e., the $\Delta\delta$ (in ppm) of each component is a function of χ^2/ν_0^2 with the following constants: I, −6000; II, +750; III, +21,000 (*12, 13*)).

thermore, we find that a twofold increase in the length of the soft pulse results in a marginal decrease in T_1/T_2; this trend is consistent in light of the theoretical value of this ratio discussed above.[2]

In conclusion, we have demonstrated that soft pulses can afford a means of inverting the signal due to the central component of a quadrupolar nucleus bound to a large macromolecule in solution, enabling one to obtain an apparent longitudinal relaxation time, T_1, of such nuclei. In the specific case of ^{27}Al NMR spectroscopy, soft pulses also result in efficient sequestering of the troublesome background ^{27}Al signal. However, under the conditions used here, one cannot selectively excite the central transition without perturbing the outer components, whose broad resonances lie in close proximity to the signal of interest. This results in a complex longitudinal relaxation behavior for the system, which undermines the extraction of physically meaningful information. Moreover, in such systems there is a practical limit to the length of the radiofrequency pulse that can be used before one can no longer invert the signal due to relaxation during the pulse. Various other factors must also be taken into account. For example, increasing B_0 will lead to an increase in T_2 (Eq. [2]), which enables one to use longer (more selective) soft pulses, yet the dynamic frequency shift, and hence the separation of the signals due to the central and outer components, decreases (Eq. [1]). Conversely, systems with increased χ should lead to an increase in the separation of the components (Eq. [1]), but also an increased linewidth (Eq. [2]), restricting the practical length of the soft pulse, and consequently the selectivity of the experiment. Furthermore, the inferior quadrupolar relaxation properties (i.e., shorter T_2) of other nuclei reported to bind to such proteins compared to ^{27}Al make this approach less practical. Hence, these studies demonstrate that, although it is possible to invert the protein-bound quadrupolar signal and obtain its T_1, the experiment is not selective enough to yield the true central transition T_1, making this a problematic route for gleaning information pertaining to the nature of the metal ion binding sites in large metalloproteins.

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[2] Inversion-recovery experiments performed at 283 K under more selective conditions using 1-ms Gaussian pulses gave T_1 values of N, 5.5 ms, and C, 3.7 ms, resulting in only slight decreases in the respective T_1/T_2 ratios.

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