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^{67}Zn QCPMG Solid-State NMR Studies of Zinc Complexes as Models for Metalloproteins

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Zinc plays a key role in the active binding site for a range of important metalloproteins.[1] For example, Zn^{2+} is important for the function of pencillamine,[2] insulin,[3] carboxypeptidase A,[4] thermolysin,[5] and phospholipase C.[6] To understand the enzymatic function of these metalloproteins, it is of interest to study the Zn^{2+} coordination environment with a variety of ligands, i.e., N, O, and S donor atoms. Information on Zn^{2+} complexation may potentially be obtained from liquid-state ^{67}Zn NMR (isotropic chemical shifts, δ_{iso}; T_1 and T_2 relaxation).[7] However, the large ^{67}Zn line width and poor receptivity will prevent useful data from being obtained on biological compounds via liquid-state NMR. Furthermore, the Zn^{2+} coordination is particularly reflected in the ^{67}Zn ($I = {}^5/_2$) quadrupole coupling, an interaction which may be obtained only indirectly from liquid-state relaxation studies. Solid-state ^{67}Zn NMR is a more direct and informative probe for the local structure but is unfortunately associated with broad line shapes due to a large quadrupole moment.

To circumvent these problems it has been popular to replace Zn^{2+} with $^{113}Cd^{2+}$ ($I = {}^1/_2$) and use empirical relations between ^{113}Cd chemical shielding anisotropy (CSA) and structure[8,9] to obtain information about metal coordination in metalloenzymes.[10] Nevertheless, ^{67}Zn NMR should be the method of choice for Zn-metalloproteins. This approach removes the potential ambiguities regarding changes in local structure induced by Cd^{2+} replacement and may be used to investigate the utility of the ^{113}Cd surrogate-probe strategy. Among the few ^{67}Zn solid-state NMR studies reported so far[11,12] one has involved the detection of a 40-kHz wide powder pattern at 11.7 T for $Zn(CH_3COO)_2{\cdot}2H_2O$[11] using the quadrupolar echo (QE) experiment.[13]

For large weight Zn^{2+} complexes with broad (50−150 kHz) second-order quadrupolar powder patterns, ^{67}Zn QE NMR may be an experimental challenge. In such cases the sensitivity must be enhanced by isotope enrichment combined with, e.g., cross polarization (CP) from ^{1}H,[14] low-temperature acquisition,[15] or sampling of the free-induction decay (FID) in the presence of a train of refocusing pulses.[16−18] Low-temperature experiments are technically difficult and may cause the sample to be in a phase different from that at ambient temperature. Similarly, CP is demanding since it requires the matching of an rf field amplitude on the ^{67}Zn channel of about 50 kHz with a 3 times larger amplitude on the ^{1}H channel to obtain an efficient spin-lock.

In this paper we demonstrate that ^{67}Zn QCPMG NMR represents a feasible approach to study Zn^{2+} coordination in model complexes for metalloenzymes. The QCPMG experiment,[18]

$$\left(\frac{\pi}{2}\right)_x - \tau_1 - (\pi)_y - \tau_2 - \mathrm{Acq.}\left(\frac{1}{2}\tau_a\right) - [\tau_3 - (\pi)_y - \tau_4 - \mathrm{Acq.}(\tau_a)]^M - \mathrm{Acq.}(\tau_d) \quad (1)$$

splits the QE line shape for the central transition into a manifold of spin−echo sidebands separated by $1/\tau_a$, where τ_a is the interpulse acquisition period with ^{1}H decoupling (M is the number of echo repetitions and τ_d an additional acquisition time to ensure full decay of the signal). Depending on the sideband separation QCPMG may enhance the sensitivity by an order of magnitude compared to QE while maintaining information on the anisotropic interactions.[18] The applicability of the method is demonstrated using ^{67}Zn-enriched zinc formate dihydrate ($Zn(OOCH)_2{\cdot}2H_2O$, **1**) and zinc diimidazole diacetate ($Zn(OOCCH_3)_2$ $(C_3H_4N_2)_2$, **2**).[19−21] These complexes are representatives of Zn^{2+} in an all-oxygen six-coordination sphere and in an 2-O, 2-N four-coordination sphere, respectively.

Figure 1 shows experimental and calculated ^{67}Zn QCPMG spectra for the two Zn sites[22,23] in **1** at 9.4 and 11.7 T. Optimized[18] δ_{iso} and quadrupole coupling (C_Q, η_Q) parameters, corresponding to the simulations in parts b and f of Figures 1, are summarized in Table 1. No convincing effects from CSA for either compound could be detected (i.e., $\Delta\sigma \leq 50$ ppm). The two Zn^{2+} sites, coordinated to six oxygens from six formate groups (Zn_f) and from four water molecules and two formate groups (Zn_w), have been tentatively assigned by comparison of ^{67}Zn CP/QE spectra for the H_2O and D_2O forms of **1** (not shown).[23] While δ_{iso} differ only slightly, the quadrupole coupling parameters differ significantly for the two sites. For both δ_{iso} and the anisotropic

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(19) **1** was synthesized by dissolving ^{67}Zn metal (90% enriched Cambridge Isotope Laboratories, MA) in equimolar amounts of concentrated formic acid and water; **2** was synthesized according to Horrocks et al.[20]
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(21) NMR experiments were performed using a home-built 9.4 T (25.04 MHz for ^{67}Zn) spectrometer and a 11.7 T Varian UNITY *plus* 500 (31.29 MHz for ^{67}Zn) spectrometer with double-tuned 5-mm probes from Doty Scientific Inc. (Columbia, SC). The QCPMG experiments employed selective $\pi/2$-pulses of 1.85 μs ($\gamma B_1/2\pi = 45$ kHz). Simulations and iterative fitting of experimental spectra were conducted on a SUN Sparc-10 as described elsewhere.[18]
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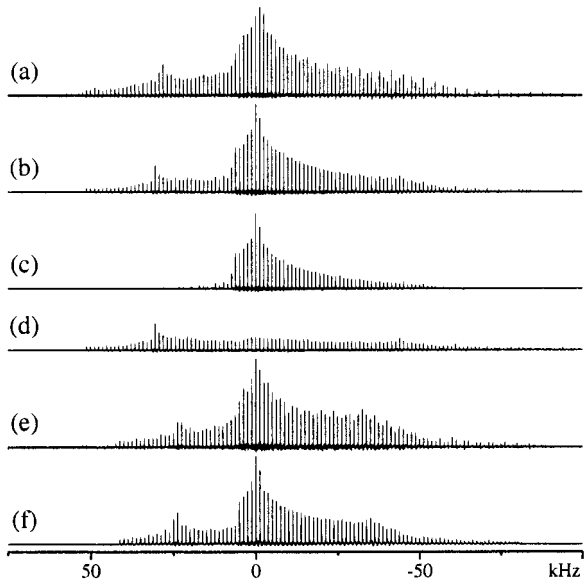

Figure 1. Experimental (a,e) and calculated (b,f) ^{67}Zn QCPMG NMR spectra of **1** at (a,b) 9.4 and (e,f) 11.7 T. The experimental spectra were acquired using the pulse scheme in eq 1 with (a) $\tau_a = 819.2$ μs, dwell time 1.6 μs, $M = 30$, recycle time 3.5 s, 70 304 transients and (e) $\tau_a = 800$ μs, dwell time 0.4 μs, M = 15, recycle time 2s, and 32 768 transients. The calculated spectra used the parameters in Table 1. (c,d) Separation of (b) into individual sideband manifolds for the (c) Zn_f and (d) Zn_w sites.

Table 1: Magnitudes of ^{67}Zn Quadrupolar Coupling Tensors and Isotropic Chemical Shifts for **1** and **2**

complex	site	C_Q (MHz)	η_Q	δ_{iso} (ppm)[a]	space group
1	Zn_f	6.05 ± 0.2	0.99 ± 0.1	-10 ± 5	$P2_1/c$[22,23]
	Zn_w	9.52 ± 0.2	0.39 ± 0.1	0 ± 5	
2		8.20 ± 0.2	0.62 ± 0.1	155 ± 5	$P\bar{1}$[20]

[a] Referenced to a 0.9 M solution of $Zn(OOCCH_3)_2 \cdot 2H_2O$.

interaction, the ^{67}Zn parameters agree qualitatively well with the ^{113}Cd δ_{iso} and CSA determined for the isomorphous cadmium formate dihydrate.[8]

The prospect of ^{67}Zn QCPMG NMR in studies of metalloproteins prompted a study of **2**, which may be regarded a model for the Zn site in thermolysin. In this enzyme Zn^{2+} is coordinated to 2-N (two histidines) and 2-O (a monodentate glutamate and water).[5] In **2** the Zn^{2+} is coordinated to 2-N (two imidazoles) and 2-O (two monodentate acetates). Experimental and calculated 9.4 and 11.7 T ^{67}Zn QCPMG spectra of **2** are shown in Figure 2. The optimized simulations (parameters in Table 1) are in good agreement with the experimental spectra at both fields. Two features are apparent from Table 1. First, for **2** C_Q is between the values for Zn_f and Zn_w in **1**. This ordering may arise for several reasons, i.e., changes in coordination number, the specific coordination geometry, donor atom type, and the empirical fact that C_Q scales with the electronegativity for the donor atoms (i.e., all-O coordinated Zn^{2+} is expected to have larger C_Q than mixed N/O coordinated Zn^{2+} in similar environment).[24] Second, δ_{iso} increases by about 155 ppm on going from 6-O Zn^{2+} coordination in **1** to the 2-O, 2-N coordinated Zn^{2+} for **2** which agrees with similar findings from ^{113}Cd NMR of Cd homologues.[8,9]

With **2** representing a model for thermolysin, it is of interest to discuss the potential of ^{67}Zn QCPMG NMR in studies of ^{67}Zn-enriched metalloproteins. The 11.7 T spectrum of **2** (Figure 2c) requires 13 h of spectrometer time. Consider a protein with a 100-fold larger molecular weight, a spectrum with half the signal-to-noise ratio (S/N) would require 32 500 h (3.7 years!). By reducing the dwell time to 0.2 μs, doubling the sideband separation, and performing the experiment at 18.7 T, the spectrometer time may be reduced by a factor of 100 h to 13.5 days. Further reduction may be achieved by combination with CP. In another approach, the 3.7 year time frame may be reduced by a factor of 5625 (ignoring an increase in T_1) by performing the QCPMG experiment at 4 K rather than 300 K (corresponding to a conservative estimate for the gain in S/N by a factor of 75). With an estimated consequence of T_1 on the experiment time by a factor of 10, this leads to an experiment time of about 60 h. This time can be reduced further by a factor of 100 using the modifications to QCPMG described above.

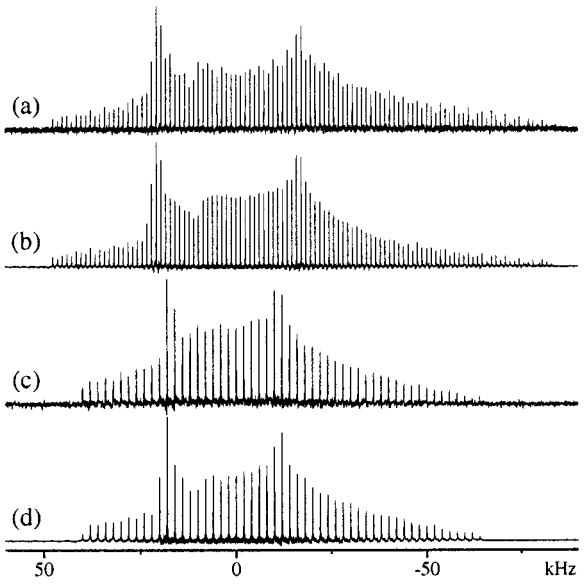

Figure 2. Experimental (a,c) and calculated (b,d) ^{67}Zn QCPMG NMR spectra of **2** at (a,b) 9.4 and (c,d) 11.7 T. The experimental spectra were recorded using (a) $\tau_a = 819.2$, dwell time 1.6 μ s, M = 30, recycle time 1.5 s, 153 896 transients and (c) $\tau_a = 500$ μs, dwell time 0.5 μs, M = 15, recycle time 2 s, and 24 000 transients. The calculated spectra used the parameters in Table 1.

In conclusion, we have demonstrated that ^{67}Zn QCPMG NMR, through its significant sensitivity enhancement compared to QE NMR, represents a powerful method in studies of zinc complexes. By the determination of relationships between the coordination geometry and the parameters for chemical shielding and quadrupole coupling tensors, we anticipate that ^{67}Zn QCPMG NMR will play a critical role in solid-state investigations of ^{67}Zn-enriched metalloproteins. Employing improved instrumentation, the sensitivity of the ^{67}Zn QCPMG experiment will be further improved by combination with CP, and acquiring the spectra at cryogenic temperatures.

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