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NMR Spectroscopy

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Solid-State ^{17}O NMR Spectroscopy of Large Protein–Ligand Complexes**

*Jianfeng Zhu, Eric Ye, Victor Terskikh, and Gang Wu**

Solid-state ^{17}O NMR spectroscopy has attracted considerable attention because of its potential as a new probe of biological structures.[1,2] One prerequisite for such applications is that the sensitivity of currently available methods for solid-state ^{17}O NMR spectroscopy is sufficient to allow direct detection of weak ^{17}O ($I = 5/2$) NMR signals from a biological macromolecule of significant size. Nearly 20 years ago, Oldfield et al.[3] reported the first set of solid-state ^{17}O NMR spectra for proteins, [$^{17}O_2$]hemoglobin and [$^{17}O_2$]myoglobin, under nonspinning (stationary) conditions in a moderate magnetic field of 8.45 T. However, as the authors noted, the poor signal-to-noise ratios obtainable at the time did not permit any detailed spectral analysis. Later, Oldfield and co-workers[4] successfully obtained solid-state ^{17}O NMR spectra for [C^{17}O]myoglobin (16.7 kDa per ligand) under conditions of magic-angle spinning (MAS) at 11.7 T. Several groups have since reported solid-state ^{17}O NMR spectroscopic studies of membrane-bound peptides.[5–7]

Herein we report the first comprehensive solid-state ^{17}O MAS NMR spectroscopic study of large protein–ligand complexes with an emphasis on addressing the sensitivity issue. In particular, we used two robust protein–ligand complexes, egg-white avidin–[$^{17}O_2$]biotin (64 kDa) and ovotransferrin–Al^{III}–[$^{17}O_4$]oxalate (80 kDa), as benchmark cases to test the detection limit at a high magnetic field of 21.14 T. We discovered that the ^{17}O spin–lattice relaxation times (T_1) in these solid protein–ligand complexes are typically on the order of several milliseconds; therefore, very rapid data collection is possible. Furthermore, we found that several sensitivity-enhancement methods uniquely suited for half-integer quadrupolar nuclei, such as double-frequency sweep (DFS),[8] rotor-assisted population transfer (RAPT),[9] and hyperbolic secant (HS) pulses,[10] can be used to obtain high-quality solid-state ^{17}O NMR spectra for large protein–ligand complexes.

Avidin is a glycoprotein isolated from hen egg white that forms a tetramer with a total molecular weight of about 64 kDa and can bind biotin molecules with extremely high affinity ($K_d = 10^{-15}$ M).[11] The ^{17}O MAS NMR spectrum of the avidin–[$^{17}O_2$]biotin complex exhibits a typical line shape arising from second-order quadrupole interactions (Figure 1).

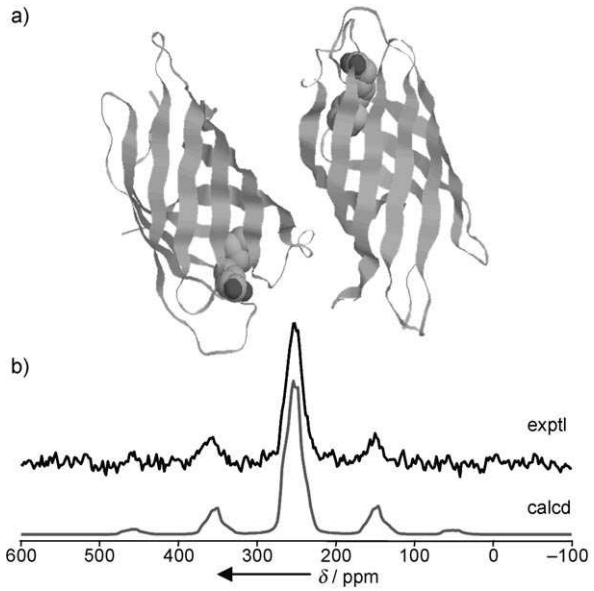

Figure 1. a) Partial crystal structure of the avidin–biotin complex (PDB entry: 1AVD).[12] Only the asymmetric unit (chains A and B) is shown. b) Experimental and simulated ^{17}O MAS NMR spectra of the avidin–[$^{17}O_2$, 18% ^{17}O]biotin complex at 21.14 T. Experimental details: approximately 20 mg of the protein was packed into an Si_3N_4 rotor (outside diameter, o.d.: 4 mm); sample spinning frequency: 12.5 kHz; single-pulse excitation with a recycle delay of 30 ms; 1.6×10^6 transients (total experimental time: 17 h). All ^{17}O chemical shifts were referenced to external liquid H_2O.

Spectral simulations suggest the following ^{17}O NMR parameters: $C_Q = 5.8$ MHz, $\eta_Q = 0.4$, $\delta_{iso} = 270$ ppm, which are typical for deprotonated carboxylate groups.[13,14] As each avidin molecule can bind four biotin molecules, the effective molecular weight of the complex is approximately 16 kDa per [$^{17}O_2$]biotin unit (a dilution factor of 1.5×10^{-2}). Because only about 20 mg of the protein and a relatively low level of ^{17}O enrichment (18%) were used, the observed signal-to-noise ratio without the use of any additional sensitivity-enhancement technique was quite encouraging.

To further test the sensitivity limit of solid-state ^{17}O NMR spectroscopic experiments at 21.14 T, we investigated a much larger protein–ligand complex, ovotransferrin–Al^{III}–

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[$^{17}O_4$]oxalate. Ovotransferrin (OTf) is a 80 kDa glycoprotein capable of binding two Al^{III} cations and two synergistic anions (e.g., carbonate or oxalate) with high affinity.[15] Thus, the effective molecular weight of the OTf–Al–oxalate complex is approximately 40 kDa per ligand (a dilution factor of 2.2×10^{-3}). Figure 2 shows the ^{17}O MAS NMR spectra of OTf–Al–oxalate at three magnetic fields. Significant improvement in spectral resolution was observed at 21.14 T. We also observed a sensitivity gain by a factor of about 2.5 when the magnetic-field strength was increased from 11.74 to 21.14 T, after taking into account all relevant effects. By using the ^{17}O NMR spectroscopic data obtained for this protein–ligand complex in solution[17] as a guide, we analyzed the experimental ^{17}O MAS spectra and obtained the following quadrupole-coupling-tensor and chemical-shift-tensor parameters: O1, $\delta_{iso}=219$ ppm, $\xi=\delta_{33}-\delta_{iso}=-160$ ppm, $\eta=(\delta_{22}-\delta_{11})/\xi=0.6$, $C_Q=5.75$ MHz, $\eta_Q=0.70$; O2, $\delta_{iso}=237$ ppm, $\xi=-160$ ppm, $\eta=0.6$, $C_Q=6.30$ MHz, $\eta_Q=0.70$; O3, $\delta_{iso}=274$ ppm, $\xi=-240$ ppm, $\eta=0.2$, $C_Q=7.70$ MHz, $\eta_Q=0.45$; O4, $\delta_{iso}=282$ ppm, $\xi=-240$ ppm, $\eta=0.2$, $C_Q=7.90$ MHz, $\eta_Q=0.35$. In the spectral simulations, we used the Euler angles $\alpha=0°$, $\beta=80°$, and $\gamma=30°$ to describe the relative orientation between the quadrupole-coupling tensor and the chemical-shift tensor[18] for all four oxygen sites, on the basis of the computational results reported by Wong et al. for oxalate–metal complexes.[19] Quite remarkably, the solid-state ^{17}O NMR parameters observed for OTf–Al–[$^{17}O_4$]oxalate are in close agreement with those found in solution,[17] which suggests that this protein–ligand complex adopts essentially the same structure in the two phases. In principle, the solid-state ^{17}O NMR spectra can be analyzed directly in the absence of solution ^{17}O NMR spectroscopic data.

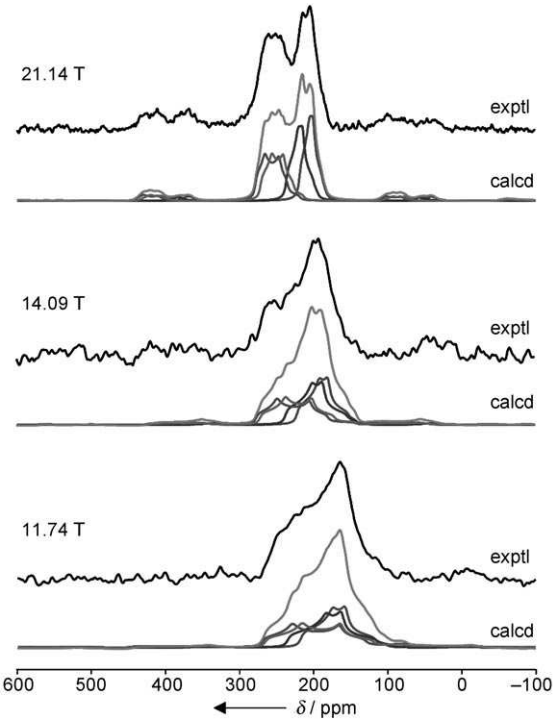

Figure 2. Experimental and simulated ^{17}O MAS NMR spectra of OTf–Al–[$^{17}O_4$]oxalate obtained at three magnetic fields. At 21.14 T, the solid protein (ca. 25 mg) was packed into a ZrO_2 rotor (3.2 mm o.d.); sample spinning frequency: 20 kHz; HS pulses (20 kHz bandwidth, 400 kHz offset); 1.0×10^6 transients; recycle time: 30 ms (total experimental time 11 h). At 14.09 T, the solid protein (ca. 75 mg) was packed into an Si_3N_4 rotor (4 mm o.d.); sample spinning frequency: 12 kHz; single-pulse excitation; 1.9×10^6 transients; recycle time: 25 ms (total experimental time: 16 h). At 11.74 T, the solid protein (ca. 75 mg) was packed into an Si_3N_4 rotor (4 mm o.d.); sample spinning frequency: 12 kHz; single-pulse excitation; 2.5×10^6 transients; recycle time: 25 ms (total experimental time: 21 h). All spectral simulations were performed with the DMFit program.[16] All three spectra were simulated with the same set of NMR parameters for each of the four oxygen sites.

To better understand the ^{17}O NMR parameters observed for OTf–Al–oxalate, we performed ab initio quantum-chemical calculations of these NMR parameters. Because the crystal structure of the OTf–Al–oxalate complex is unknown, we used the crystal structure of human serum transferrin–Fe^{III}–oxalate (PDB entry: 1RYO)[20] as a starting point to build a molecular cluster model to mimic the oxalate-binding pocket in OTf–Al–oxalate, including all hydrogen-bonding interactions. This initial model did not yield satisfactory computational results. Most likely, the geometry of the oxalate ligand in OTf–Al–oxalate is somewhat different from that in 1RYO, which is not unexpected.

Indeed, close inspection of 1RYO revealed some structural features that are inconsistent with other crystallographic data observed for small Al–oxalate complexes. For example, the C–O bonds that coordinate to the Al center are usually longer than noncoordinating C–O bonds (1.28 versus 1.22 Å).[21] However, the crystal structure 1RYO[20] shows the opposite trend (1.23 versus 1.26 Å). For this reason, we decided to refine the geometry of the oxalate ligand by using our experimental ^{17}O NMR parameters together with experimental solution-state ^{27}Al and ^{13}C NMR spectroscopic data from the literature (^{27}Al: $\delta_{iso}=1.5$ ppm, $P_Q=4.3$ MHz; ^{13}C: $\delta_{iso}=168.4$, 164.6 ppm).[22] We also recorded solid-state ^{13}C and ^{27}Al NMR spectra for the same OTf–Al–oxalate sample (data not shown); the results were in excellent agreement with the solution NMR spectroscopic data. We performed a partial geometry optimization for the oxalate ligand and the hydrogen atoms involved in hydrogen bonding to the oxalate ligand at the B3LYP/6-31G(d,p) level while keeping all other heavy atoms in the cluster model fixed in place. The cluster model contained a total of 181 atoms. After this partial geometry optimization, the bond lengths of the coordinating and noncoordinating C–O bonds of the oxalate ligand were 1.27/1.32 and 1.24/1.23 Å, respectively, and the two Al–O bond lengths were 1.96 and 1.98 Å. The hydrogen-bonding environment in the oxalate-binding pocket is illustrated in Figure 3.

On the basis of this partially optimized structure, we computed ^{17}O, ^{27}Al, and ^{13}C NMR parameters (both quadrupole parameters and chemical shifts). The agreement between the experimental and computational results was reasonably good (Figure 4), which further supports the validity of the refined model. It does not appear that multinuclear ^{17}O, ^{27}Al, and ^{13}C NMR parameters have previously been used *simulta-*

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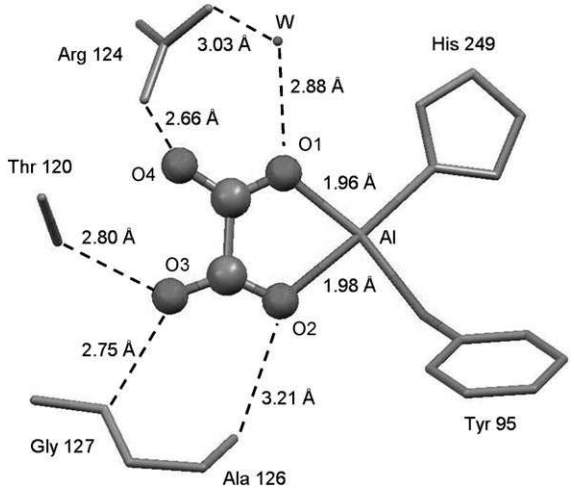

Figure 3. Hydrogen-bonding environment of the oxalate-binding pocket in OTf–Al–oxalate. A water molecule (W) is hydrogen-boned to Arg 124 and O1. Two axial ligands (Tyr 188 and Asp 63) at the Al center are omitted for clarity. The geometry of the oxalate ligand was optimized at the B3LYP/6-31G(d,p) level.

neously to aid structural refinement of a protein-bound ligand molecule. We believe that this aspect of "NMR crystallography"[23] should be further explored.

The OTf–Al–oxalate complex (40 kDa per ligand) is the most dilute protein system investigated so far by solid-state ^{17}O NMR spectroscopy. This benchmark case enables us to assess the sensitivity limit of solid-state ^{17}O NMR spectroscopy for the study of proteins. The OTf–Al–oxalate sample (ca. 25 mg protein) used at 21.14 T contained only approximately 30 μg of [$^{17}O_4$, 50 % ^{17}O]oxalate. Because the ^{17}O spin–lattice relaxation time, T_1, is quite short for this solid protein (< 4 ms), we were able to collect ^{17}O NMR spectroscopic data very rapidly (e.g., with a recycle delay of 30 ms). In fact, we found that solid proteins generally have very short ^{17}O T_1 values (Table 1). Moreover, the ^{17}O T_1 value decreased as the applied magnetic field was increased, which suggests

Table 1: Experimental ^{17}O NMR spin–lattice (T_1) and spin–spin (T_2) relaxation times of two protein–ligand complexes in the solid state at different magnetic fields.

Protein complex	Relaxation time [ms]					
	11.74 T		14.09 T		21.14 T	
	T_1	T_2	T_1	T_2	T_1	T_2
OTf–Al–oxalate	6.6	2.4	5.1	2.1	< 4	1.9
avidin–biotin	3.5	0.5	2.8	0.7	1.0	0.5

the presence of very fast local motion in solid proteins, most likely as a result of the presence of methyl groups in the vicinity of the ligand. In contrast, the ^{17}O T_1 value for a crystalline sample of sodium oxalate is on the order of 1 s (250 times longer). To further improve sensitivity, we used the HS pulses to selectively invert the satellite transitions.[10] This approach routinely produces an additional gain of a factor of about 2 in sensitivity enhancement (a factor of 4 in time

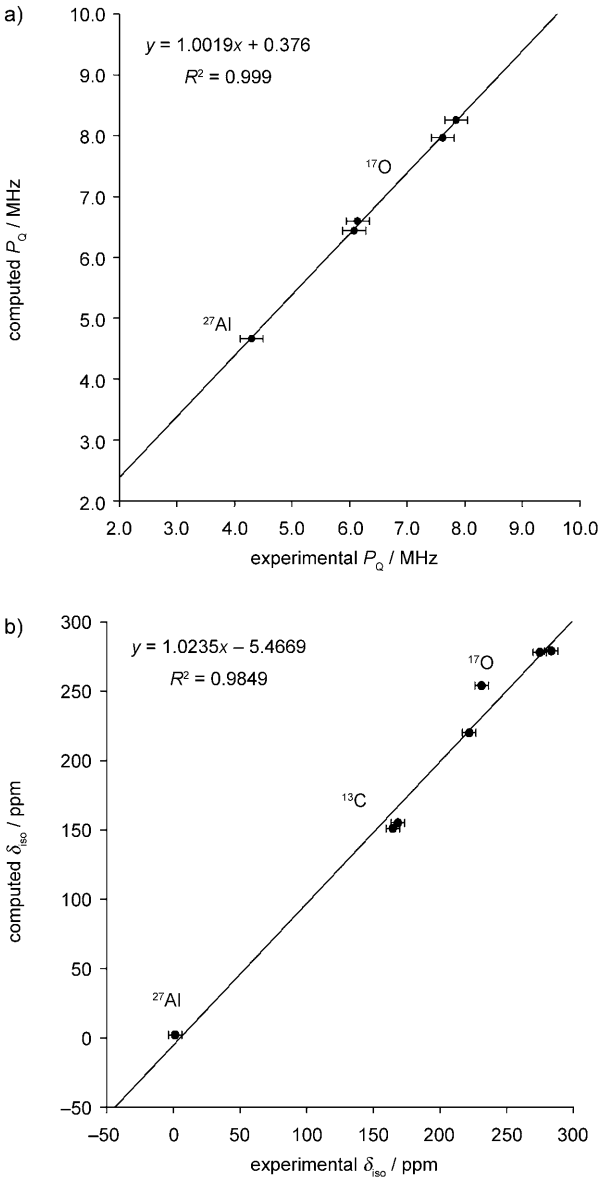

Figure 4. Comparison between the computed and experimental NMR parameters a) P_Q and b) δ_{iso} for the refined model of OTf–Al–oxalate. In (a), the quadrupole parameter is defined as $P_Q = C_Q(1+\eta_Q^2/3)^{1/2}$. The uncertainty in the experimental P_Q and δ_{iso} values was estimated to be ± 0.2 MHz and ± 5 ppm, respectively.

saving) in comparison with single-pulse excitation. Other sensitivity-enhancement methods, such as RAPT and DFS, yielded similar results.

On the basis of these new solid-state ^{17}O NMR spectroscopic results, we can conclude that one should be able to obtain high-quality ^{17}O MAS NMR spectra at 21 T for protein–ligand complexes as large as 300 kDa per ligand with 90 % ^{17}O enrichment and a data-acquisition time of 48 h. This kind of sensitivity will make it possible to apply solid-state ^{17}O NMR spectroscopy to many important proteins. We hope that this report will encourage others to consider solid-state ^{17}O NMR spectroscopy as a viable technique for probing protein–ligand interactions.

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Experimental Section

Chicken egg-white avidin (Lot. 026K7044), (+)-biotin 4-nitrophenyl ester (99% purity), and chicken ovotransferrin (Lot. 107K7022) were purchased from Sigma–Aldrich. [$^{17}O_2$]Biotin was prepared by the base-catalyzed hydrolysis of (+)-biotin 4-nitrophenyl ester in CH_3OH/CH_2Cl_2 with Na[^{17}O]H (45% ^{17}O). [1,2-$^{13}C_2$]Oxalic acid (99%) was purchased from Cambridge Isotope Laboratories, Inc. Sodium [$^{17}O_4$]oxalate was prepared by dissolving oxalic acid (50 mg) in ^{17}O-enriched H_2O (70% ^{17}O; 0.3 mL), heating the solution at 50–55 °C overnight, and then neutralizing the solution with dry NaOH. The ^{17}O-enriched water was recovered on a vacuum line. The avidin–[$^{17}O_2$]biotin complex was prepared in phosphate buffer (pH 7.4). The OTf–Al^{III}–oxalate complex was prepared as follows: OTf (ca. 80 mg) was dissolved in H_2O (0.75 mL), and $Al(NO_3)_3$ and $Na_2C_2[^{17}O_4]$ were added to an Al/oxalate/OTf molar ratio of approximately 2:2:1. The protein concentration was determined by absorption at 280 nm ($\varepsilon = 91\,200\,M^{-1}\,cm^{-1}$). KCl was also added to the protein solution to a final concentration of 150 mM. The final pH value of the protein solution was adjusted to 7.5. The amount of free ligand in the protein sample was monitored by solution ^{17}O NMR spectroscopy. In both protein samples, the amount of free ligand present in solution was negligible. Solid proteins were obtained by drying the protein solution with a flow of N_2 gas at room temperature.

Solid-state ^{17}O NMR spectroscopic experiments were performed on Bruker Avance 500 (11.74 T), Avance 600 (14.09 T), and Avance II 900 (21.14 T) NMR spectrometers operating at 67.8, 81.3, and 122.0 MHz, respectively. For solid protein samples, the ^{17}O spin–lattice relaxation times were determined by using the saturation-recovery method, and the ^{17}O transverse relaxation times were measured by using a rotor-synchronized spin-echo pulse sequence. All quantum-chemical calculations were performed on Sun Fire 25000 servers at the High Performance Computing Virtual Laboratory (HPCVL) of Queen's University. Each of the servers is equipped with 72 dual-core UltraSPARC-IV+ 1.5 GHz processors with 576 GB of RAM. All calculations were performed with the Amsterdam Density Functional (ADF) software package.[24] Double-zeta (DZ) basis sets and the zeroth-order regular approximation (ZORA) for relativistic corrections were used for all shielding calculations. The equation $\delta = \sigma_{ref} - \sigma$ was used to convert the computed shielding constants (σ) into chemical shifts (δ). We used $\sigma_{ref} = 287.5$,[25] 185.4,[26] and 580.3 ppm for ^{17}O, ^{13}C, and ^{27}Al shielding scales, respectively. A value of 580.3 ppm for ^{27}Al was obtained for $[Al(H_2O)_6]^{3+}$, the geometry of which was optimized at the B3LYP/6-311++G(d,p) level.

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