

Ion Solvation by Channel Carbonyls Characterized by ^{17}O Solid-State NMR at 21 T

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Multiple technologies and methodologies have been used in the characterization of structure, dynamics, kinetics, and energetics of facilitated ion transport, and still there is much that we do not fully understand about how proteins achieve this process.^{1,2} By taking advantage of very high magnetic fields we report here on the development of a new approach through the application of ^{17}O solid-state nuclear magnetic resonance (NMR) spectroscopy to probe ion binding to the gramicidin A (gA) carbonyl oxygens.

Ion selectivity and high conductance rates in the K^+ channels take advantage of the backbone carbonyl oxygens^{3,4} that provide much of the solvation environment for K^+ when it binds to the channel. This principle also applies to the gA channel, which functions as a monovalent cation selective channel.² Previously, this cation–oxygen interaction was detected indirectly by monitoring the perturbation of either the amide ^{15}N chemical shift (CS)^{5,6} or the isotropic and anisotropic carbonyl ^{13}C CS in the same peptide plane.^{7,8}

Although ^1H , ^{13}C , and ^{15}N NMR spectroscopies have been well developed to study the structure, function, and dynamics of proteins and nucleic acids, biological ^{17}O NMR studies are rare,^{9–15} despite the fact that oxygen is a key participant in many biological functions, such as enzyme–substrate interactions, nucleic acid–base pairing, ion ligation, etc. Intrinsically large CS anisotropy and quadrupolar interactions render ^{17}O a sensitive probe to explore such functions. However, biological ^{17}O NMR has suffered from low sensitivity mainly due to the large quadrupolar interactions of ^{17}O spins ($S = 5/2$), which result in broad lines. The advent of very high magnetic fields minimizes the second-order broadening of the ^{17}O resonances and result in both resolution and sensitivity enhancement. Here, for the sake of simplifying the spectral interpretation, we have chosen to single-site label gA.

Gramicidin A is a 15-residue polypeptide with an alternating sequence of D- and L-amino acids: HCO-L-Val1-Gly2-L-Ala3-D-Leu4-L-Ala5-D-Val6-L-Val7-D-Val8-L-Trp9-D-Leu10-L-Trp11-D-Leu12-L-Trp13-D-Leu14-L-Trp15-NHCH₂CH₂OH.² In lipid membranes dimeric gramicidin A forms a right-handed β -helix in which a 4.5 Å pore accommodates ions and a single file of water molecules.^{16,17} Previous NMR studies found that the carbonyl oxygen of D-Leu10 is one of the three carbonyls⁷ involved in the ion binding site at each end of the channel and perturbation of ^{15}N anisotropic chemical shift was observed when gA was predominantly occupied with two cations.⁶ Initially, $^{17}\text{O}_2$ -D-leucine was prepared from H₂¹⁷O (70% ^{17}O enrichment, CIL, Andover, MA) by acid-catalyzed exchange at high temperature, followed by Fmoc protection.¹⁸ ^{17}O -[D-Leu10]-gA was then synthesized by solid-phase peptide synthesis following the previous procedure.⁶ Estimated from the FT-ICR mass spectrum of the final product, the ^{17}O enrichment of ^{17}O -[D-Leu10]-

gA was approximately 57%. This reduction from 70% is primarily due to isotope dilution during ^{17}O exchange of the amino acid.

Parts a and b of Figure 1 display the ^{17}O magic angle spinning (MAS) and static NMR spectra of a lyophilized ^{17}O -[D-Leu10]-gA sample. The gA powder spectrum was simulated (in-house developed MatLab program) with a set of parameters in reasonable agreement with the amide ^{17}O data from small peptides.^{13,19} The fitting of the ^{17}O MAS and static spectra provide the ^{17}O isotropic CS, $\delta_{\text{iso}} = 285 \pm 2$ ppm; the quadrupolar coupling (QC) constant, $\chi = 8.0 \pm 0.1$ MHz; the asymmetry parameter, $\eta = 0.3 \pm 0.04$; the principal components of the ^{17}O CS tensors, $\delta_{11} = 490 \pm 10$, $\delta_{22} = 400 \pm 10$, and $\delta_{33} = -35 \pm 10$ ppm; and the Euler angles $\alpha = 0 \pm 10^\circ$, $\beta = 90 \pm 2^\circ$, and $\gamma = 72 \pm 4^\circ$ that define the relative orientation of CS and QC tensors.¹⁹ These NMR tensor elements are illustrated in Figure 1 with an approximate alignment of the tensors to the molecular frame based on the previously reported orientations in amides.^{19,20}

The ^{17}O NMR spectrum of ^{17}O -[D-Leu10]-gA uniformly aligned in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers shows a single peak at 470 ppm (Figure 2a) when the bilayer normal is parallel to the external magnetic field, B_0 . When the bilayer normal is perpendicular to B_0 , a single resonance at 180 ppm appears in the spectrum (Figure 2b). These two frequencies define the δ_{\parallel} and δ_{\perp} components of the CS tensor motionally averaged by global rotation about the channel axis. With additional characterization of the ^{17}O tensor in the gA channel conformation it will be possible to use the 470 ppm CS as an orientational and structural restraint for the Leu10 carbonyl site.

Figure 3 compares the ^{17}O NMR spectrum of the aligned ^{17}O -[D-Leu10]-gA to that in the presence of K^+ . At 2.4 M KCl, at least 80% of the gA channels are doubly occupied, i.e., one ion per monomer.^{6,21} Previously, ^{15}N anisotropic CS data suggested that the ion binding site in each monomer represented a shallow energy minimum in which the ion interacted with three different carbonyl oxygens nonsimultaneously. Indeed, if the interaction strength was the same with each of the oxygens, the data could be interpreted as fractional occupancies for the interaction with each carbonyl.⁶ Such an analysis for K^+ suggested that the fractional occupancy with D-Leu10 was approximately 40%. Here, the time-averaged influence of K^+ on the ^{17}O anisotropic CS results in a frequency of 430 ppm and a shift of 40 ppm (Figure 3b), compared to an 8 ppm shift for the ^{15}N anisotropic CS.⁶ Such a large shift in ^{17}O CS demonstrates that ^{17}O is a sensitive probe for studying ion binding in channels.

In summary, the ^{17}O tensor element magnitudes and orientations from a single-site labeled macromolecule were shown to be in close agreement with published model compound data. Despite a 30 ppm line width for a resonance from an aligned sample, the 525 ppm breadth of the anisotropic CS tensor results in the observed

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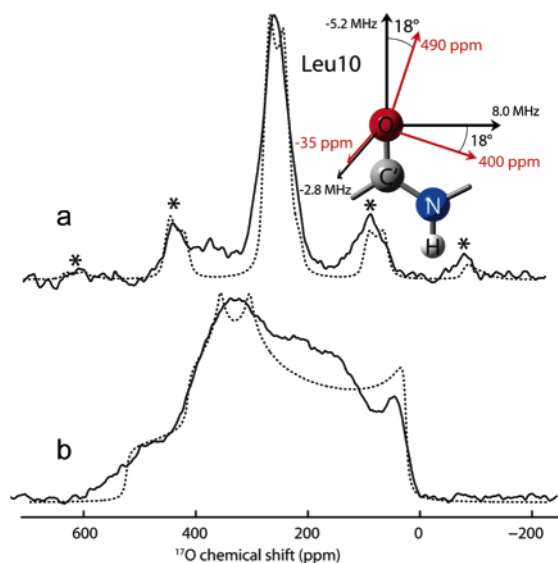


Figure 1. Proton-decoupled ^{17}O (a) MAS and (b) static NMR spectra (solid lines) of lyophilized ^{17}O -[D-Leu10]-gA. Simulated spectra shown as dashed lines. The ^{17}O chemical shift of H_2O using a rotor synchronized spin-echo sequence ($\tau_{180^\circ} = 2^* \tau_{90^\circ} = 4.0 \mu\text{s}$, spinning rate ~ 20 kHz and ~ 10 mg of gA powder in a 2.75 mm rotor with 26k transients, a repetition rate of 3 s and ^1H B_1 (decoupling) = 70 kHz. Natural abundance ^{17}O from the spinning rotor contributes to the signals at 380 ppm. Asterisks indicate the spinning sidebands. The static spectrum was obtained at 122 MHz using a spin-echo sequence ($\tau_{180^\circ} = 2^* \tau_{90^\circ} = 2.3 \mu\text{s}$, $10 \mu\text{s}$ echo period), ~ 15 mg of gA powder with 60k transients, repetition rate of 1 s and ^1H B_1 (decoupling) = 75 kHz using the NMFLL ultra-wide bore 900 MHz magnet.

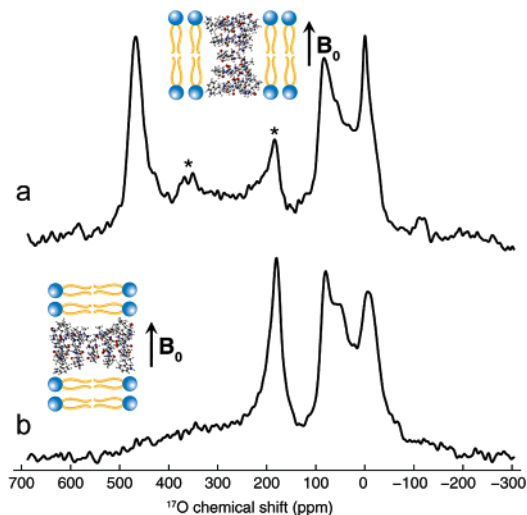


Figure 2. Proton-decoupled ^{17}O NMR spectra of ^{17}O -[D-Leu10]-gA uniformly aligned in DMPC bilayers with a peptide:lipid ratio of 1:16. The sample was oriented between $30\text{-}\mu\text{m}$ thick glass slides following the literature procedure.⁶ Both spectra were obtained from the 900 MHz spectrometer at 313 K with a home-built static probe using a spin-echo sequence ($\tau_{180^\circ} = 2^* \tau_{90^\circ} = 4.0 \mu\text{s}$, $10 \mu\text{s}$ echo period), ~ 8 mg of gA with $\sim 30\text{k}$ transients each, repetition rate of 3 s and ^1H B_1 (decoupling) = 30 kHz. The ^{17}O CS of H_2O was referenced as 0 ppm. Signals ranging from 0 to 100 ppm correspond to the backbone signal from lipids and water. Signals marked with an asterisk may result from unaligned portions of the sample (i.e. powder pattern singularities at 185 and 360 ppm). The aligned sample was oriented in such a way that the magnetic field was (a) parallel to or (b) perpendicular to the bilayer normal.

frequency being a high-quality orientational restraint for the gA structure. Finally, and most importantly, the ^{17}O resonance is highly sensitive to the presence of cations. Indeed, the shift in ^{17}O anisotropic CS for resonances in polycrystalline peptides (unpublished results) is 2–3 times the observed shift here. This result further

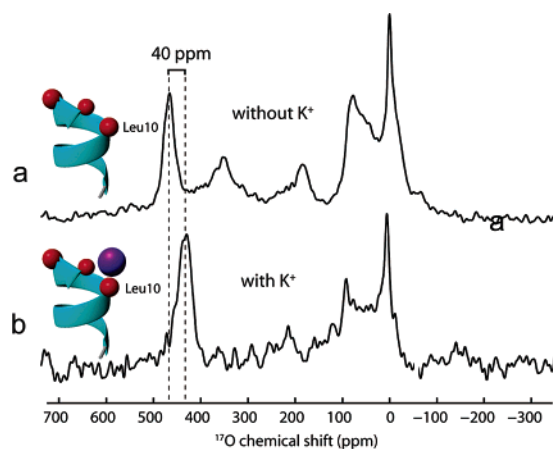


Figure 3. ^{17}O NMR spectra of ^{17}O -[D-Leu10]-gA uniformly aligned in DMPC bilayers. (a) The sample (8 mg of gA) was rehydrated with H_2O . (b) The sample (4 mg of gA) was rehydrated with 2.4 M KCl. Both spectra were obtained at 298 K and other conditions as in Figure 2 with 27k and 36k transients, respectively, using repetition rate of 2s.

supports the model for the ion-binding site in gA where the observed shift is a time averaged interaction over three carbonyl interacting sites. Such a time-averaged interaction with these three carbonyls generates a stepwise dehydration of the cation as it enters the channel and results in the ion maintaining considerable entropy while in the binding site. These high-field ^{17}O results open a new approach for characterizing ion channels and many other important biological functions performed by macromolecules.

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