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$^{15}N,^{1}H$ Heteronuclear Correlation NMR of Gramicidin A in DMPC-d_{67}

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NMR is a powerful technique for unraveling the structure of biopolymers, as long as they are in solution where the rapid tumbling of molecules averages out the anisotropies such as chemical shift and dipole−dipole interactions. As a result of the fine resolution, the resonances can be easily assigned and correlated. Membrane-associated proteins represent a unique class of biopolymers whose structure and function are dependent on the presence of the hydrophobic interior of a lipid bilayer where total isotropic averaging can never take place. Micellar dispersions of small membrane polypeptides have been used to mimic the amphipathic nature of lipid membranes while allowing orientational averaging for high-resolution spectra; however, because of their high curvature, micelles are criticized for not accurately representing the membrane bilayer.

Because of the need for methods to study membrane protein structure, there have been many advances in the application of solid-state NMR to the study of membrane-associated proteins in recent years.[1] In some cases, the orientational distribution of the polypeptides is narrowed by stacking the bilayers between glass plates; the anisotropic interactions are then used to deduce the orientation of the amide bonds in the polypeptide relative to the normal of the bilayer, as described by Opella and co-workers.[2] In other cases, the anisotropies are simply removed by magic angle spinning (MAS) and by RF decoupling schemes, yielding liquidlike high-resolution spectra. Selected anisotropic interactions are then reintroduced by a wide variety of methods and are used to obtain structural constraints.[1] In either case, it is clear that the ability to achieve well-resolved signals is indispensable for using NMR as a technique to study membrane protein structure.

The dominant interaction for nuclei in polypeptides dispersed in multilamellar lipid phases is the strong dipolar coupling among the hydrogens. However, it has been shown that in the context of rapid axial reorientation within the membrane, the dipolar broadening becomes inhomogeneous and the protein exhibits sharp ^{1}H signals if it is submitted to MAS at a rate faster than the intermediate time scale motions.[3] Within the limits of the MAS speeds, this approach can provide proton signals for small membrane proteins with resolutions approaching those of solution protein samples. Even so, the residual interactions are sufficiently strong to severely restrict, through fast relaxation processes, the ability to perform *J*-coupling-based high-resolution experiments that often require delays on the order of several tens of milliseconds.

In the present communication, we propose a heteronuclear correlation experiment based on cross-polarization, which demonstrates the dispersion and resolution of NMR signals from the amide groups in membrane polypeptides. For this, we used gramicidin A (gA), a model membrane polypeptide dimer with a conformation that is highly sensitive to the environment, incorporated into the fluid lamellar phase of chain and headgroup deuterated dimyristoylphosphatidylcholine (DMPC-d_{67}).

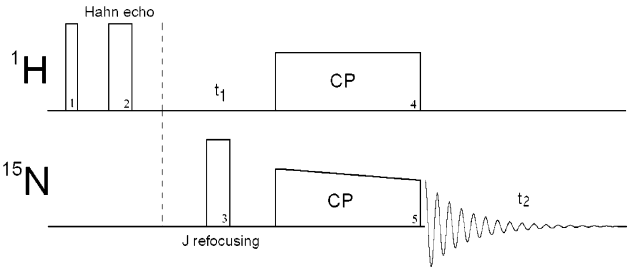

Figure 1. Pulse profile of CROPSY. Pulses 1 and 2 form a short ^{1}H echo synchronized with a rotor period to simultaneously refocus the time-dependent local field and the spin−spin interactions at the start of the indirect evolution period t_1. Pulse 3 refocuses the evolution of heteronuclear J_{HN} coupling during t_1. The ^{1}H coherence transfer to ^{15}N is optimized with the straight and ramped (100%−90%) cross-polarization 200 μs pulses 4 and 5, respectively. The phase cycling included $\phi1 = [(x)_2(-x)_2(y)_2(-y)_2]_2$, $\phi2 = (y,-y)_2(-x,x)_4(-y,y)_2$, $\phi3 = (x)_{16}$, $\phi4 = [(-x)_4(x)_4]_2$, $\phi5 = (x)_4(-x)_4(y)_4(-y)_4$, $\phi_{rcvr} = [(x)_2(-x)_2]_2[(-y)_2(y)_2]_2$.

^{15}N-labeled gA was obtained from *B. brevis* (ATCC 8085) cultivated on a modified asparagine-glycerol minimal medium (mAGM medium) on the basis of a previously published procedure.[4] The gA peptide was then separated on a reverse-phase HPLC C_{18} column (10 × 100 mm, Waters). The gA fraction eluted as the largest absorbance peak at 280 nm at 20 min, using a linear gradient solvent mixture (methanol/H_2O 70%/30% to 100%/0% in 30 min) at a flow rate of 10 mL/min.

The peptide and deuterated phospholipids were weighed and combined in a molar ratio of 1:20, then codissolved in a methanol−chloroform mixture (1/1 v/v), which was removed by rotary evaporation followed by overnight vacuum evaporation. The sample was hydrated by adding 1 "dry weight" equiv of 50 mM potassium phosphate buffer (pH = 6.0). The paste was homogenized, introduced into a 4 mm Si_3N_4 rotor with long Aurum caps, and allowed to stabilize for a prolonged period under experimental conditions until an equilibrium state was reached.

The entire set of NMR experiments was carried out on a Bruker 54 mm Ultrashield-600 spectrometer (Karlsruhe, Germany) equipped with a Doty Scientific $^{1}H/X/Y$, XC4-NB, 4 mm MAS probe (Columbia, South Carolina) tuned at 600.13 MHz for ^{1}H and 60.82 MHz for ^{15}N. Typical ^{1}H and ^{15}N 90° pulse lengths were 5.8 and 7.5 μs, respectively. Cross-polarization was achieved with spin-lock fields of about 33 kHz. Figure 1 shows the profile of the cross-polarization spectroscopy[5] (CROPSY) pulse sequence reminiscent of the WISE experiment[6] but geared here toward high resolution. The MAS rate was 12 kHz, and the sample was maintained at a temperature of 55 °C.

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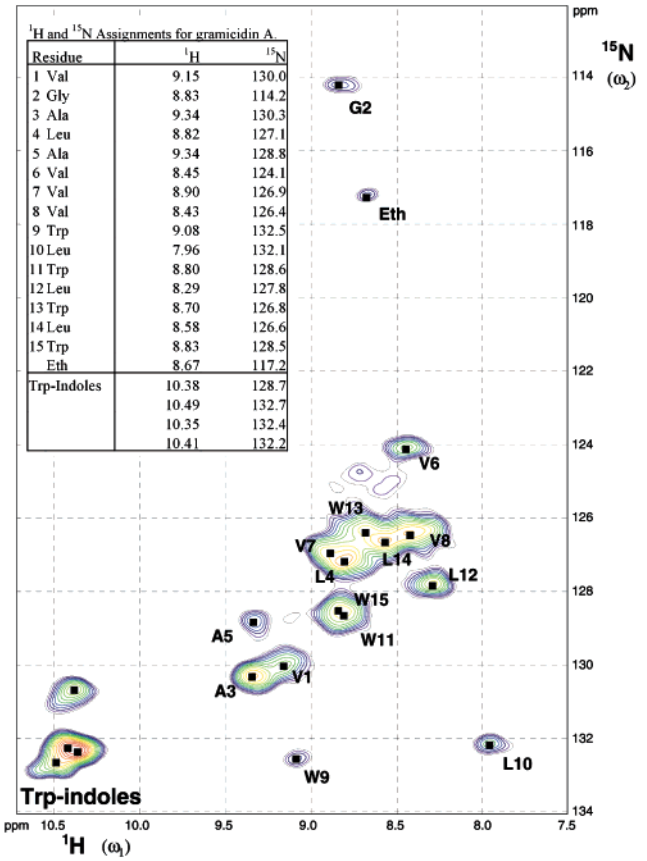

Figure 2. 2D-CROPSY of ^{15}N-gA. The experiment required 256 t_1 increments, with 256 scans per increment and 2k points in t_2. The recycling delay was 0.9 s. The spectrum was processed with a Gaussian function (LB = −15, GB = 0.08) in ω, and with linear prediction and a squared sinebell function in ω_1. Black squares are positioned on the maximum of each resolved peak and extrapolated for overlapping peaks. Assignments are based on similarity with the HSQC spectrum of gA in SDS and are summarized in the inset table.

The 2D CROPSY spectrum is shown in Figure 2. There is sufficient dispersion to recognize 19 peaks corresponding to the 20 H−N pairs for gA, with only two strongly overlapping peaks (W11/W15). Yet, eight peaks present no overlap at all. The full-widths at half-height were 41 and 164 Hz in the ^{15}N and ^{1}H dimensions, respectively. The intensity of the peaks shows important variations, which may partially be due to the dependence of the transfer on the N−H bond order parameter and on its orientation relative to the local membrane normal.

Although a definitive assignment of the resonances cannot be achieved from this experiment alone, it is nonetheless of interest to compare the chemical shifts to those obtained by high-resolution NMR experiments, such as HSQC (heteronuclear single-quantum correlation spectroscopy) on ^{15}N-gA in soluble form. The conformationally polymorphic gA was previously shown to form a stable right-handed $\beta^{6.3}$-helix in sodium dodecyl sulfate (SDS) dispersion[7] and a right-handed antiparallel $\beta^{7.2}$-double-helical dimer in a methanol/chloroform mixture (MeOH/$CHCl_3$) with cesium thiocyanate.[8] Furthermore, it partly adopts the left- and right-handed $\beta^{4.4}$-helix in trifluoroethanol (TFE)[9] and the left-handed $\beta^{6.3}$-helix conformation in dimethyl sulfoxide (DMSO).[10] Only the latter reports ^{15}N assignments, so we completed the assignments for the other three solvent media (SDS, TFE, and MeOH/$CHCl_3$) using analogously prepared samples. To compare the amide chemical shifts from the CROPSY experiment with those reported in HSQC for gA in different forms, we minimized the root-mean-square difference (rmsd) summed over all backbone sites (Table 1). These results indicate that the relative shifts and range of dispersion of the resonances in both dimensions closely match those observed in HSQC of gA dispersed in SDS, but agree only poorly with similar results in TFE, DMSO, and MeOH/$CHCl_3$. The tentative assignment shown in the inset to Figure 2 is based on the closeness of fit of the chemical shifts to those obtained in SDS.

Table 1. Minimum Root Mean Square Difference, Summed over All Backbone Amide Sites, for the ^{1}H and ^{15}N Chemical Shifts of Gramicidin A in DMPC (Obtained by CROPSY) Relative to Those Observed for Different Soluble Conformations by HSQC

structure	reference	environment	^{1}H	^{15}N
right-handed N-to-N single-stranded helical dimer ($\beta^{6.3}$)	6	SDS	0.136	0.34[a]
right-handed antiparallel double-helical dimer ($\beta^{7.2}$)	7	MeOH/$CHCl_3$	0.301	2.06[a]
left- and right-handed $\beta^{4.4}$-helix	8	TFE	0.235	2.21[a]
left-handed $\beta^{6.3}$-helix	9	DMSO	0.331	3.32

[a] The ^{15}N chemical shifts for these systems were obtained from HSQC experiments on ^{15}N-gA samples prepared as described in the references.

These results strongly support the premise that micellar dispersions can adequately mimic the amphipathic nature of a membrane bilayer even for a conformationally sensitive peptide like gA. Moreover, they are evocative of the right-handed $\beta^{6.3}$-helix conformation of gA dispersed in DMPC in a nonoriented sample under MAS, as reported previously in oriented systems.[11] More importantly, we have shown that this experiment, when applied to small peptides associated with a nonoriented fluid membrane, offers sufficient peak resolution to simultaneously distinguish resonances from most residues. Accordingly, this experiment could be used in combination with protocols based on cross-relaxation or dipolar recoupling to collect distance and orientational restraints involving N−H groups.

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Supporting Information Available: A table containing the ^{15}N and ^{1}H chemical shift assignments for all 20 nitrogen sites of gA in SDS micelles, TFE, DMSO, and MeOH/$CHCl_3$ used to calculate rmsd values (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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