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High-Speed Magic Angle Spinning Solid-State ^{1}H Nuclear Magnetic Resonance Study of the Conformation of Gramicidin A in Lipid Bilayers

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ABSTRACT One- and two-dimensional solid-state ^{1}H nuclear magnetic resonance spectra of gramicidin A incorporated in a dimyristoylphosphatidylcholine membrane have been obtained with use of high-speed magic angle spinning. By rotating the sample at 13 kHz, it is possible to observe signals in the ^{1}H spectra between 6.0 and 9.0 ppm attributable to the aromatic protons of the tryptophan residues and the formyl group proton of gramicidin A. Two-dimensional solid-state COSY spectra provided information for the peak assignments. Moreover, changes in the ^{1}H spectra have been observed as a function of the co-solubilization solvent initially used to prepare the samples and therefore as a function of the conformation adopted by gramicidin A. Three organic solvents have been used: trifluoroethanol, a mixture of methanol/chloroform (1:1 v/v), and ethanol. The conformational interconversion of gramicidin A from the double helix conformation to the channel structure for the sample prepared from ethanol was confirmed by following the time evolution of the proton spectra.

INTRODUCTION

High resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy has become a powerful method for protein structure determination in solution. However, this method has not yet been applied to membrane protein systems because the strong homogeneous dipole-dipole broadening between neighboring hydrogen nuclei, which is averaged to zero by rapid isotropic reorientation of molecules in solution, is not completely averaged away in anisotropic nonspinning membrane systems and leads to spectral line widths of 5–25 kHz (10–50 ppm depending on the magnetic field strength) (Bloom et al., 1977; MacKay, 1981). This broadening is comparable to, or even larger than, the chemical shift dispersion obtained at the highest magnetic field available. Therefore, the spectral details found in high resolution ^{1}H NMR cannot be seen in static (nonspinning) experiments on membrane systems.

Several techniques have been used to attenuate the dipolar interactions in solid samples. The use of a combination of rotation with a multiple-pulse sequence (CRAMPS) (Bronnimann et al., 1988) achieved until now the best results for the enhancement of the resolution. However, the resolution obtained is still modest; moreover, the sensitivity is greatly reduced because of the required sampling between pulses. Another approach to solid-state ^{1}H NMR spectroscopy is the isotopic dilution of protons by deuterons (McDermott et al., 1992; Zheng et al., 1993). By increasing the distances between protons, dipolar interactions are greatly reduced, and spectra with resolution comparable to or better than that observed with the CRAMPS technique can be obtained. The problem of isotopic dilution, however, is to achieve a 90% deuterium labeling of the sample in order to optimize the resolution.

These two methods can be applied to various molecules. However, if the molecules studied already undergo rapid axially symmetric reorientation, then high resolution spectra can be obtained by the use of magic angle spinning (MAS) alone (Oldfield et al., 1987; Davis et al., 1995). In 1987–1988, it was discovered that MAS at a rate of only 3–4 kHz was sufficient to obtain well resolved ^{1}H spectra from multilamellar dispersions of phospholipids in water (Oldfield et al., 1987; Forbes et al., 1988). The line width obtained, 0.03–0.05 ppm, represents a narrowing of the spectrum by a factor of ~300. The most important motion that permit MAS to narrow the homogeneously broadened ^{1}H NMR spectra of lipid multilamellar dispersions is the rapid axial diffusion of the phospholipid about its long axis. Rapid axial diffusion has the same effect as rapid axial rotation: it projects the intramolecular dipolar interactions onto the diffusion axis. The dipolar broadening then depends only on the orientation of the local bilayer normal relative to the magnetic field, scaling as $(3\cos^2\theta\text{-}1)/2$ where θ is the angle between the bilayer normal and the magnetic field. In this manner, the homogeneous dipolar broadening is converted into an inhomogeneous broadening, which can be effectively averaged by MAS.

Although it is possible to obtain well resolved lipid ^{1}H spectra from multilamellar dispersions, no identifiable peptide signals can be observed at spinning speeds of ~3–4 kHz for small peptides, such as gramicidin A, incorporated in phospholipid bilayers. Initially it was believed that the peptide was not reorienting rapidly enough about the bilayer normal to average out the homogeneous interactions. However, several studies (Datema et al., 1986; Prosser et al., 1994; Prosser and Davis, 1994; Davis, 1988; Macdonald and Seelig, 1988; North and Cross, 1993) have indicated that gramicidin does indeed diffuse sufficiently rapidly

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about the local bilayer normal, with correlation times in the range of 10^{-9} to 10^{-7} s. On the other hand, it can be shown that there are additional intermediate time scale motions that broaden the peptide resonances. ^{2}H NMR relaxation studies of gramicidin A in oriented samples of dilauroylphosphatidylcholine (Prosser et al., 1994; Prosser and Davis, 1994) have demonstrated that the wobble of the diffusion axis with respect to the local bilayer normal, which for that system has a correlation time of $\sim 6 \times 10^{-6}$ s, is probably the most significant cause of line broadening of the ^{1}H resonances of the peptide. Therefore, it should be possible to observe ^{1}H NMR MAS spectra of membrane peptides, but in order to do so it is necessary to spin the sample at a spinning speed faster than that required for phospholipid multilamellar dispersions (Davis et al., 1995).

In this paper we demonstrate by the use of high-speed MAS one- and two-dimensional solid-state ^{1}H NMR that it is possible to study small peptides incorporated in phospholipid membranes. More specifically, the conformation of gramicidin A, a 15 amino acid hydrophobic peptide that has been widely used as a model for the hydrophobic part of intrinsic membrane proteins (Urry, 1984), has been investigated in multilamellar dispersions of dimyristoylphosphatidylcholine (DMPC). In addition, because various techniques such as circular dichroism (Killian et al., 1988a), NMR spectroscopy (Lograsso et al., 1988; Killian et al., 1988b; Killian, 1992), high-pressure liquid chromatography (Bañó et al., 1991), and vibrational spectroscopy (Bouchard and Auger, 1993) showed that the conformation of gramicidin A is highly dependent on the organic solvent initially used to co-dissolve the peptide with the lipid, the proton NMR spectra have been recorded for three different co-solubilization solvents: trifluoroethanol, a mixture of methanol/chloroform (1:1 v/v), and ethanol. The results indicate that different conformations of gramicidin A can be detected using high-speed MAS solid-state ^{1}H NMR. In addition, the results clearly demonstrate the possibilities of the technique for conformational and dynamical studies of small peptides incorporated in lipid bilayers.

EXPERIMENTAL PROCEDURES

Materials

Gramicidin A and DMPC were obtained from Fluka Chemika-Biochemica (Ronkonkoma, NY) and Sigma Chemical Co. (St. Louis, MO), respectively, and were used without any further purification. Trifluoroethanol was purchased from Sigma, and methanol and chloroform were purchased from Fisher Scientific (Pittsburgh, PA). The salts used in the buffer were of analytical grade.

Preparation of samples

Samples of DMPC/gramicidin A were prepared in a 10:1 molar ratio by co-dissolving the appropriate amount of peptide and lipid in the organic solvent (1 ml). In order to obtain homogeneous peptide/lipid systems, the samples were incubated at 52°C for 1 h and shaken on a vortex mixer at least a few times during the incubation cycle. After the incubation, the organic solvents were evaporated with a nitrogen stream followed by high vacuum pumping overnight to ensure complete evaporation of the solvents. The samples were then hydrated (50 wt %) with a Na_2HPO_4 buffer prepared in D_2O at pD 7.0 and submitted to several cycles of heating (52°C), vortex-shaking, and cooling (0°C); ~30 μl of sample was used for each NMR experiment.

NMR measurements

High speed MAS ^{1}H spectra were recorded on a Bruker ASX-300 solid-state spectrometer (Bruker Spectrospin, Milton, Ontario, Canada) operating at a proton frequency of 300 MHz. A 4-mm MAS probehead (Bruker Spectrospin) was used for all experiments. The one-dimensional spectra (8 K data points) were acquired with a standard one-pulse sequence at 60°C. Six hundred forty scans were recorded for each spectrum. The 90° pulse length was 4.1 μs, and the recycle delay was 5 s. Additional increase in the recycle delay did not change the intensity of the spectra. The sample spinning speed was set to 13.000 kHz and was controlled to within ± 0.002 kHz with use of a Bruker MAS controller (Bruker Spectrospin). A 5-Hz line broadening was applied to all one-dimensional spectra. The chemical shifts, expressed in part per million, are reported relative to the signal of external tetramethylsilane (TMS) at 0 ppm. Spin-lattice relaxation times (T_1) were measured by the inversion recovery technique using a recycle delay of 5 s. Relaxation times were obtained from a single exponential fit of the data. The two-dimensional spectrum was obtained using a COSY (correlation spectroscopy) sequence. Five hundred twelve spectra (1024 data points) were acquired in the first dimension, and the sweep width was set to 20 ppm in both dimensions. One hundred seventy-six scans were recorded for each spectrum. A shifted sine-bell window function was used in both dimensions in the processing of the two-dimensional data set. The peaks in the two-dimensional spectrum are obtained in the absolute value mode, and the final spectrum was symmetrized before plotting.

RESULTS AND DISCUSSION

Solid-state proton NMR spectra of gramicidin A in lipid bilayers

Fig. 1 shows the MAS solid-state proton NMR spectra at 60°C of pure DMPC and of the DMPC/gramicidin A system initially prepared from trifluoroethanol. Both spectra were recorded at a spinning speed of 13.000 ± 0.002 kHz. In the spectrum of the DMPC/gramicidin A system, we can observe signals between 6.0 and 9.0 ppm. In contrast, in the pure DMPC spectrum no signal can be observed in this region. Therefore we can conclude that the signals observed in the DMPC/gramicidin A spectrum between 6.0 and 9.0 ppm are caused exclusively by the gramicidin A protons. Other resonances that can be attributed to gramicidin A are also present underneath some of the lipid resonances.

Experiments performed at slower spinning speeds indicate that the line widths of the resonances observed between 6.0 and 9.0 ppm are strongly dependent on the spinning speed, the signal becoming significantly broader at slower speeds. Therefore, high-speed MAS (in this case, 13 kHz, which was the highest spinning speed possible on our system for aqueous samples) provides substantial line narrowing of the proton resonances (Davis et al., 1995).

On the basis of high resolution liquid NMR studies of gramicidin A (Arseniev et al, 1985; Pascal and Cross, 1992), the spectral region between 6.0 and 9.0 ppm can be assigned to the aromatic protons of the tryptophan

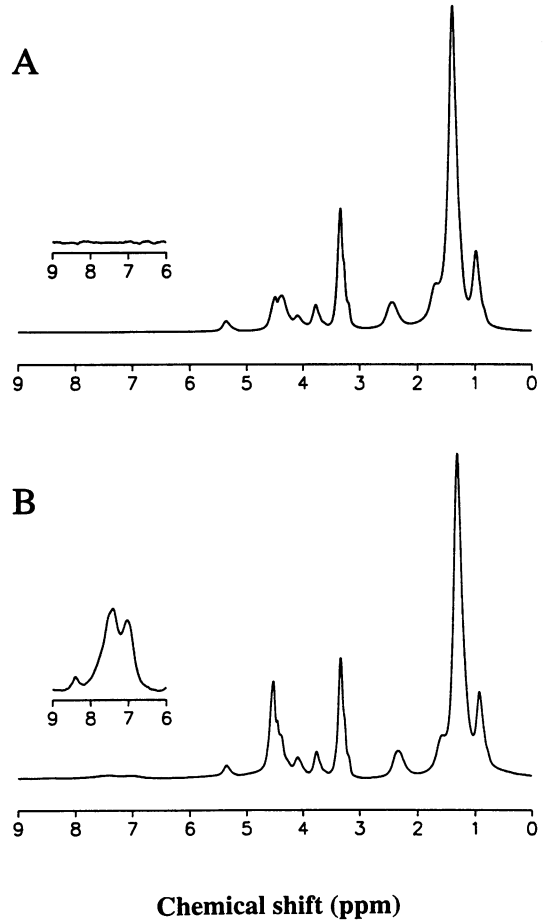

FIGURE 1 (*A*) MAS solid-state ^{1}H spectra at 60°C of pure DMPC and (*B*) DMPC/gramicidin A system initially prepared from trifluoroethanol. The spinning speed was set to 13.000 ± 0.002 kHz.

residues (resonances at 7.0 and 7.4 ppm) and to the formyl group proton of gramicidin A (resonance at 8.4 ppm). In addition, we have recorded a two-dimensional COSY NMR spectrum of the DMPC/gramicidin A system in order to confirm the peak assignments of gramicidin A. Fig. 2 shows the contour plot of the solid-state COSY spectra in the spectral region between 6.0 and 9.0 ppm for the DMPC/gramicidin A system initially prepared from trifluoroethanol. The presence of cross-peaks between the resonances at 7.0 and 7.4 ppm and the absence of cross-peaks with the resonance at 8.4 ppm support the idea that the low frequency resonances are caused by the aromatic protons of gramicidin A. Therefore, even though the complete assignment of the protons in this spectral region is not possible, a distinction between the formyl group proton and the indole ring protons is clearly possible. On the other hand, the width of the resonances at 7.0 and 7.4 ppm also reflects the distribution of isotropic chemical shifts for the indole protons of the four tryptophan residues in each gramicidin molecule.

Integration of the signal between 6.0 and 9.0 ppm indicates that the full intensity expected in this spectral region is

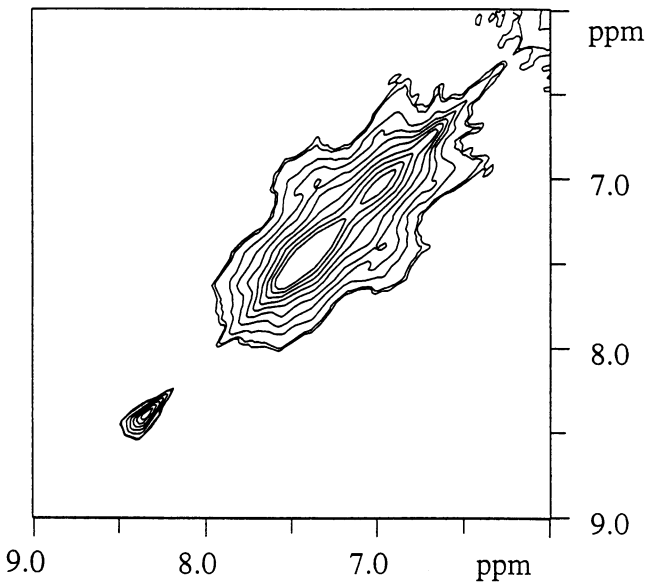

FIGURE 2 Contour plot of the solid-state ^{1}H COSY spectrum in the spectral region between 6.0 and 9.0 ppm for the DMPC/gramicidin A system initially prepared from trifluoroethanol. The spectrum was recorded at 60°C.

seen relative to the lipid signal at 2.36 ppm. In addition, we have measured the spin-lattice relaxation times (T_1) for the tryptophan protons and the formyl group proton of gramicidin A. T_1 values of 749 ms and 742 ms have been obtained for the tryptophan protons, and a T_1 value of 738 ms has been obtained for the formyl group proton. These results indicate that the recycle delay of 5 s used in the experiments is long enough to observe the full intensity of the gramicidin resonances.

Conformation of gramidicin A in lipid bilayers

Fig. 3 shows the MAS solid-state proton NMR spectra at 60°C between 6.3 ppm and 8.7 ppm for the DMPC/gramicidin A systems initially prepared from three different organic solvents: ethanol, a mixture of methanol/chloroform (1:1 v/v), and trifluoroethanol. For the three systems, the

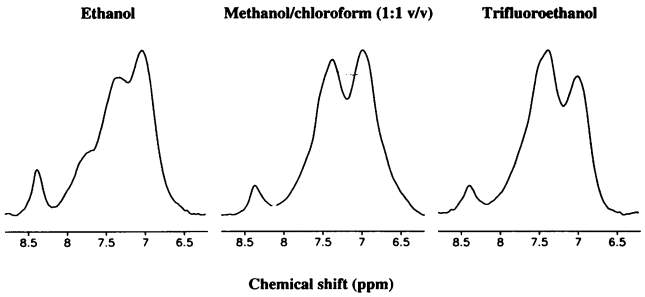

FIGURE 3 MAS solid-state ^{1}H spectra at 60°C between 6.3 ppm and 8.7 ppm for DMPC/gramicidin A systems initially prepared from the three organic solvents: ethanol, a mixture of methanol/chloroform (1:1 v/v), and trifluoroethanol.

full intensity expected in this spectral region indicates that the relaxation delay of 5 s is always longer than five T_1 for the three systems. In addition, the spectra clearly show significant changes in the line shape and chemical shifts of the gramicidin A signals in the three systems.

The changes observed in the spectra presented in Fig. 3 can be explained by the fact that the conformation adopted by gramicidin A varies with the organic solvent initially used in the preparation of the samples. Trifluoroethanol was originally suggested as a good co-solubilization solvent because of the monomeric nature of gramicidin A molecules in trifluoroethanol solution (Urry et al., 1972). In fact, when trifluoroethanol is used as the co-solubilization solvent, gramicidin A is incorporated in lipid bilayers as an amino terminal to amino terminal hydrogen-bonded dimer in the single-stranded $\beta^{6.3}$ helical conformation (Killian et al., 1988a,b; Killian, 1992; Ketchem et al., 1993). In this conformation, the four tryptophan residues (at positions 9, 11, 13, and 15) are located near the bilayer surface, close to the lipid carbonyl groups (Bouchard and Auger, 1993). By contrast, when ethanol (in which gramicidin A tends to form intertwined dimers) is used, it incorporates in the nonchannel state, and the channel structure is slow to appear (Killian et al., 1988a; Lograsso et al., 1988; Bouchard and Auger, 1993). In such systems, the dominant conformation of gramicidin A is the double helix in which the tryptophan residues are located uniformly along the peptide axis, therefore having chemical environments different from those obtained in a $\beta^{6.3}$ helix. When the co-solubilization solvent is a methanol/chloroform (1:1 v/v) solution, a mixture of conformations is obtained, including the channel and the double helix conformations (Killian et al., 1988a; Lograsso et al., 1988; Bouchard and Auger, 1993).

The chemical shifts obtained for the systems prepared from trifluoroethanol, which favors the channel conformation, and from ethanol, which favors the double helix conformation, are given in Table 1, part 1. For the system prepared from trifluoroethanol, the formyl proton is located at 8.39 ppm, whereas two major resonances can be observed for the tryptophan protons at 7.02 and 7.42 ppm. For the system prepared from ethanol, three resonances can be observed at 7.05, 7.36, and 7.72 ppm for the tryptophan protons, whereas the formyl proton is still present at 8.38 ppm.

These results can be compared with those obtained from solution NMR spectra of gramicidin A in both the channel conformation (incorporated into sodium dodecyl-d_{25} sulfate micelles) (Arseniev et al., 1985) and the double helix conformation (gramicidin A species 4 in dioxane) (Pascal and Cross, 1992). The chemical shifts for the tryptophan and formyl group protons obtained in these studies are listed in Table 1, part 2. When gramicidin A is in the channel structure, the formyl group proton is located at 8.35 ppm, and the indole proton resonances are distributed between 6.90 ppm and 7.55 ppm (Arseniev et al., 1985). This dispersion is much smaller than that obtained for gramicidin A in the double helix conformation in dioxane (Pascal and Cross, 1992) and for the multilamellar dispersions prepared in ethanol, in which resonances up to 7.9 ppm can be detected. Therefore, the chemical shifts measured in the solid-state NMR spectra of gramicidin A incorporated in different conformations in DMPC bilayers are similar to those measured by solution NMR. The comparison is particularly interesting for the channel conformation, inasmuch as both the solution and the solid-state NMR measurements have been performed in lipid environments. Therefore, our results clearly demonstrate that solid-state proton NMR spectra are sensitive to structural changes of small peptides incorporated in lipid bilayers.

TABLE 1 ^{1}H NMR chemical shifts of the tryptophan and the formyl group protons in gramicidin A

Part 1. Solid-state NMR results as a function of the co-solubilization solvent initially used in the preparation of DMPC/gramicidin A samples

	Chemical shift (ppm) in	
Residue	Trifluoroethanol	Ethanol
Formyl	8.39	8.38
L-Trp (9, 11, 13, 15)	7.02:7.42	7.05:7.36:7.72

Part 2. High-resolution NMR results for two gramicidin A conformations

	Chemical shift (ppm) in	
Residue	Channel conformation*	Double helix conformation‡
Formyl	8.35	
L-Trp 9	C_2H 7.03: C_4H 7.42: C_5H 6.90: C_6H 7.05: C_7H 7.36	C_2H 7.164: C_4H 7.895: C_5H 6.858: C_6H 6.987: C_7H 7.223
L-Trp 11	C_2H 7.18: C_4H 7.36: C_5H 6.91: C_6H 7.04: C_7H 7.34	C_2H 6.672: C_4H 7.362: C_5H 6.781: C_6H 6.946: C_7H 7.153
L-Trp 13	C_2H 7.32: C_4H 7.50: C_5H 6.90: C_6H 7.05: C_7H 7.41	C_2H 6.895: C_4H 7.657: C_5H 6.887: C_6H 6.987: C_7H 7.175
L-Trp 15	C_2H 7.42: C_4H 7.55: C_5H 6.97: C_6H 7.14: C_7H 7.48	C_2H 6.887: C_4H 7.571: C_5H 7.044: C_6H 7.082: C_7H 7.257

*Arseniev et al., 1985. Gramicidin A incorporated into sodium dodecyl-d_{25} sulfate micelles.

‡Pascal and Cross 1992. Gramicidin A species 4 in dioxane.

Time evolution of the conformation of gramidicin A in lipid bilayers

In order to study the conformational interconversion of gramicidin A from the double helix conformation to the channel structure (Killian et al., 1988a; Lograsso et al., 1988), we have monitored the spectrum of the DMPC/gramicidin A sample prepared from ethanol as a function of time. The results are shown in Fig. 4. The bottom right part of the figure shows the superposition of the spectrum recorded after 385 min and the spectrum of DMPC/gramicidin A system initially prepared from trifluoroethanol. The spectra shown in Fig. 4 clearly indicate that

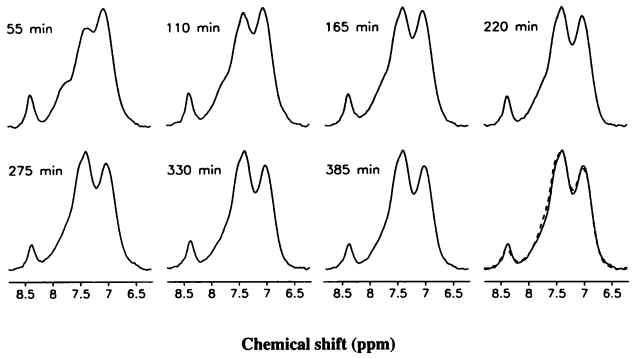

FIGURE 4 Time evolution of the spectra of the DMPC/gramicidin A sample initially prepared from ethanol. The spectra were recorded at 60°C. The bottom right part of the figure shows the superposition of the spectrum taken after 385 min (*solid line*) and the spectrum of DMPC/gramicidin initially prepared from trifluoroethanol (*dashed line*).

gramicidin A undergoes a conformational transition toward the channel structure. Even though channel formation from intertwined helical species is difficult because it requires unwinding of the intertwined helix (Killian et al., 1988a; Lograsso et al., 1988), the results demonstrate that this transition pathway is possible and that the rate of interconversion at 60°C is less than 7 h. It is interesting to note that inasmuch as the conformation of gramicidin A tends toward the $\beta^{6,3}$ helix even if the co-solubilization solvent initially used in the preparation of the samples does not favor the channel structure, long experiments such as the measurements of two-dimensional spectra or the determination of T_1 values cannot be performed on the nonchannel structures.

CONCLUSIONS

The novel possibility of using high-speed MAS to obtain solid-state proton NMR spectra of small peptides incorporated in lipid membranes has been demonstrated in this paper. The results clearly show that high-speed MAS can provide sufficient line narrowing to average the intermediate time scale motions responsible for the broadening of the peptide resonances in the proton spectra of gramicidin-lipid systems. With the resolution achieved in our spectra, signals that can be attributed to the formyl group and to the indole ring protons can be distinguished in the spectra of gramicidin A incorporated into DMPC bilayers. In addition, it has been demonstrated that the resonances of the tryptophan indole protons vary significantly for the different conformations adopted by gramicidin A in lipid bilayers, indicating that solid-state proton NMR is sensitive to structural changes of small peptides incorporated in lipid membranes.

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