High-Resolution Mono- and Multidimensional Magic Angle Spinning ^{1}H Nuclear Magnetic Resonance of Membrane Peptides in Nondeuterated Lipid Membranes and H_2O

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ABSTRACT High-speed (14 kHz) solid-state magic angle spinning (MAS) ^{1}H NMR has been applied to several membrane peptides incorporated into nondeuterated dilauroyl or dimyristoylphosphatidylcholine membranes suspended in H_2O. It is shown that solvent suppression methods derived from solution NMR, such as presaturation or jump-return, can be used to reduce water resonance, even at relatively high water content. In addition, regioselective excitation of ^{1}H peptide resonances promotes an efficient suppression of lipid resonances, even in cases where these are initially two orders of magnitude more intense. As a consequence, ^{1}H MAS spectra of the peptide low-field region are obtained without interference from water and lipid signals. These display resonances from amide and other exchangeable ^{1}H as well as from aromatic nonexchangeable ^{1}H. The spectral resolution depends on the specific types of resonance and membrane peptide. For small amphiphilic or hydrophobic oligopeptides, resolution of most individual amide resonance is achieved, whereas for the transmembrane peptide gramicidin A, an unresolved amide spectrum is obtained. Partial resolution of aromatic ^{1}H occurs in all cases. Multidimensional ^{1}H-MAS spectra of membrane peptides can also be obtained by using water suppression and regioselective excitation. For gramicidin A, F2-regioselective 2D nuclear Overhauser effect spectroscopy (NOESY) spectra are dominated by intermolecular through-space connectivities between peptide aromatic or formyl ^{1}H and lipid ^{1}H. These appear to be compatible with the known structure and topography of the gramicidin pore. On the other hand, for the amphiphilic peptide leucine-enkephalin, F2-regioselective NOESY spectra mostly display cross-peaks originating from though-space proximities of amide or aromatic ^{1}H with themselves and with aliphatic ^{1}H. F3-regioselective 3D NOESY-NOESY spectra can be used to obtain through-space correlations within aliphatic ^{1}H. Such intrapeptide proximities should allow determination of the conformation of the peptide in membranes. It is suggested that high-speed MAS multidimensional ^{1}H NMR of peptides in nondeuterated membranes and in H_2O can be used for studies of both peptide structure and lipid-peptide interactions.

INTRODUCTION

Most specific functions of biological membranes are carried out by proteins embedded in the lipid bilayer or bound to its surface. Moreover, the activity of many bioactive hydrophobic or amphiphilic peptides involves their interaction with the membrane lipid phase, which generates specific conformations. One of the most challenging goals of current structural biophysics is the determination of the tridimensional structure of such membrane proteins and peptides. Aside from crystallographic methods, several NMR approaches have been designed in the past few years for such a purpose, both in solution and in the solid state. Recent solution NMR studies of membrane peptides and proteins rely upon detergent solubilization of the protein or peptide and application of ^{1}H-detected multidimensional experiments (for a review see Henry and Sykes, 1994). This requires that the native polypeptide conformation be maintained in the solubilized state, a feature that may be difficult to assess in many cases. Solid-state NMR has the advantage of being performed on proteins and peptides in real membrane environment. Recent studies in this field have involved the measurement of peptide or protein internuclear distances by rotational resonance or REDOR (for a review see Smith, 1993) as well as peptide bond angle determination on oriented samples (for a review see Smith and Peersen, 1992; Opella et al., 1994; see also Sanders and Landis, 1995). Unlike solution NMR, until recently, solid-state NMR studies have mainly used low magnetogyric ratio nuclei such as ^{15}N (Ketchem et al., 1993; Bechinger et al., 1993; North et al., 1995), ^{13}C (Creuzet et al., 1991; Smith and Borman, 1995), and ^{2}H (Prosser et al., 1994; Prosser and Davis, 1994), selectively incorporated in the protein or peptide, rather than the ubiquitous and sensitive ^{1}H. Indeed, the applicability of solid-state NMR ^{1}H-detected experiments was seemingly precluded because of the very large homogeneous ^{1}H linewidths of protein or peptide resonances associated with the limited averaging of homonuclear dipolar interactions by restricted diffusion in membrane medium.

Recently Davis et al. (1995) and Bouchard et al. (1995) have described an approach that at least partially overcomes this limitation. Inspired by the pioneering work of Oldfield and collaborators on membrane lipids (Oldfield et al., 1987; Forbes et al., 1988a,b), these authors showed that high-speed magic angle spinning (MAS) could be used to aver-

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age a major part of the dipolar interactions and were able to observe resolved aliphatic and aromatic resonances of intrinsic peptides in membranes with relatively narrow linewidths. This opens the future prospect of applying this method to the structural study of membrane peptides in situ. It is noteworthy that to selectively observe the ^1H resonances of a peptide within a lipid membrane environment, these studies have used either deuterated lipids (Davis et al., 1995) or, alternatively, hydrogenated lipid with low lipid-to-peptide ratios (Bouchard et al., 1995). Moreover, both studies were performed on membrane samples suspended in ^2H_2O, a feature that precluded the observation of peptide-exchangeable ^1H.

In the present study we describe several further developments of this high-speed ^1H MAS NMR approach to peptides in membranes. One improvement is the application of water suppression methods commonly used in solution spectroscopy (for a review see Guéron et al., 1991). It is shown that such techniques permit the recording of ^1H MAS spectra of membrane-bound peptides in H_2O and the observation of resonances from exchangeable ^1H including amide resonances. Such resonances are likely to be important for the structural determination of membrane peptides. A second improvement is the use of selective excitation methods that make it possible to obtain MAS ^1H spectra of membrane peptides in nondeuterated lipid membranes, even at relatively high lipid-to-protein ratios. This is an extension of a technique that we introduced recently to obtain ^1H spectra of membrane peptides in nondeuterated detergents (Seigneuret and Lévy, 1995). We show that these water and lipid suppression schemes can be used to obtain 1D spectra as well as multidimensional NOESY-type spectra of membrane peptides in nondeuterated lipid membrane suspended in H_2O. The obtained resolution is evaluated for several types of membrane-bound peptides, as well as the potential application of 2D and 3D experiments for the study of peptide structure and lipid-peptide interactions.

EXPERIMENTAL PROCEDURES

Preparation of samples

1,2-Dimyristoyl-sn-glycerol-3-phosphocholine (DMPC) and 1,2-dilauroyl-sn-glycerol-3-phosphocholine (DLPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Gramicidin A (Dubos preparation) and leucine-enkephalin were purchased from Sigma (France), and myristoyl-Lys-Arg-Thr-Leu-Arg (myr KRTLR) and carbobenzoxy-D-Phe-Phe-Gly (zfFG) were from Bale-Biochimie (France). NMR samples were prepared by dissolution of the lipid and peptide in trifluoroethanol, drying under a flow of nitrogen, overnight removal of residual solvent in vacuo, and addition of the required amount of H_2O or ^2H_2O. The ^2H exchanged gramicidin A sample was prepared similarly, except that the peptide was initially dissolved in perdeuterated ethanol and incubated for 24 h at 50°C and that the final membrane sample was again incubated for 24 h at 50°C to generate the pore conformation (Killian et al., 1988). The samples (80 μl) were then homogenized with a teflon pestle, frozen and thawed several times, and packed by centrifugation into 4-mm Bruker (Wissembourg, France) zirconium oxide MAS rotors. Occasional pH measurements with a microelectrode inside the samples indicated values of ~6.

NMR experiments

^1H-NMR spectra were recorded at 400 MHz on a Bruker AMX 400 spectrometer equipped with a Bruker MAS controler and a Bruker 4DB variable-temperature MAS probe accommodating a ^2H external field-frequency lock. The temperature was regulated to less than 0.1°C and the spinning speed to less than 10 Hz. For all experiments, a 90° hard pulse of 7.3 μs and a relaxation delay of 1 s were used. Control experiments indicated that the use of a longer relaxation delay led to an increase in peptide ^1H resonance intensities of less than 10%. Single-pulse 1D spectra were recorded in 256 or 1024 scans using a 15–18 ppm spectral width and a 4k complex points time domain. 2D NOESY spectra were recorded with standard phase cycling (Neuhaus and Williamson, 1989) in the States-TPPI mode (Simorre and Marion, 1990), using spectral widths and time domains of 15–18 ppm and 1k complex points in F2 and 12–15 ppm and 128 complex points in F1 with 256 scans per increment. Three-dimensional NOESY-NOESY spectra were recorded according to the method of Boelens et al. (1989) and in the States-TPPI mode with spectral widths and time domains of 15 ppm and 512 complex points in F3 and 10 ppm and 48 complex points in both F1 and F2 with 16 scans per increment. Water suppression was effected by either low-power (0.06 kHz) selective irradiation of the H_2O resonance during the relaxation delay or by using a jump-return sequence (Plateau and Guéron, 1982) as the read pulse. Selective excitation of the peptide amide-aromatic region was performed by using a DANTE-z/IBURP-2 scheme (occasionally combined with jump-return) as the read pulse (Roumestand et al., 1995; Seigneuret and Lévy, 1995), with an RF power of 10 kHz and an interpulse delay ranging from 120 to 180 μs, depending on the spectral width of the selected spectral region. WALTZ-16 ^1H-decoupled ^{31}P NMR spectra of the samples were also routinely recorded at zero spinning speed, using the same NMR setup to check that a bilayer configuration was preserved in all cases. Spectra were processed with Felix 2.3 (Biosym Technologies, France) or NMRPipe (Delaglio et al., 1995). The 3D time-domain data were extended to 64 complex points in both indirect dimensions using forward-backward linear prediction (Zhu and Bax, 1992). The 2D and 3D time domain data were multiplied with shifted sinebells and zero-filled once in each dimension before Fourier transformation. A local baseline correction of the amide-aromatic region was applied in the F2 dimension of 2D spectra using the FLATT method (Güntert and Wuthrich, 1992) and in the F3 dimension of 3D spectra using a fourth-order polynomial. A t1 noise suppression routine (Manoleras and Norton, 1992) was occasionally applied to 2D spectra. Chemical shifts were referenced relative to external trimethylsilyl(2,2,3,3-$^2H_4$)propionate.

RESULTS

We have used high-speed (14 kHz) ^1H MAS NMR under a variety of spectroscopic conditions to study several membrane peptides incorporated into multilamellar nondeuterated phospholipid membranes suspended in H_2O. The purpose of this work was to evaluate four different topics: 1) the applicability of water suppression methods for the observation of peptide-exchangeable ^1H resonances; 2) the efficiency of selective excitation methods for the observation of peptide resonances without interference from lipid resonances; 3) the resolution achievable in the amide-aromatic region of several membrane peptides; 4) the potential usefulness of multidimensional experiments based upon the above improvements.

Water suppression in MAS ^{1}H NMR of a membrane peptide in bilayers

Fig. 1, *A* and *B*, shows the ^{1}H MAS spectrum of the intrinsic peptide gramicidin A incorporated into nondeuterated DLPC multilayers (mole ratio 1:20) suspended, respectively, in H_2O and ^2H_2O (50% lipid by weight). The spectra were recorded with a standard single-pulse sequence at 50°C and at a spinning speed of 14 kHz. Under such conditions, in H_2O, the lipid resonances are only 2.5–10 times less intense than the water signal and can therefore be observed without much interference from the latter. Fig. 1 *C* shows a spectrum recorded in H_2O under similar conditions, except that selective irradiation during the relaxation delay (i.e., presaturation) was used to suppress the water resonance. Good suppression of the water peak is obtained. A significant reduction of the intensity of the DLPC resonances is also observed as compared to Fig. 1 *A*. We have found that this limited decrease in intensity occurs invariably and is not specifically related to H_2O irradiation, inasmuch as it is also observed in ^2H_2O, even when an empty resonance position is irradiated. It appears to be specific to MAS experiments and is not observed in solution NMR experiments on vesicles or proteins performed with presaturation. Therefore this process likely arises from a limited spectral diffusion of saturation promoted by sample rotation. This is not unexpected, because sample rotation promotes a periodic modulation of the resonance position of each spin packet. Apart from this effect, the lipid spectrum looks similar to that of an identical sample in ^2H_2O recorded without presaturation (Fig. 1 *B*). In both the ^2H_2O and H_2O spectra, at the used lipid-to-protein ratio, aliphatic and H_α peptide resonances are completely masked by the lipid resonances. However, resonances are apparent in the low-field region at high magnification that do not occur with DLPC alone and are therefore attributable to gramicidin A. As already reported by Bouchard et al. (1995), the low-field resonances observed in ^2H_2O originate from gramicidin A formyl and tryptophan aromatic ^{1}H. Furthermore, it can be observed that additional resonances are present in H_2O. These arise from peptide-exchangeable ^{1}H. This indicates that such ^{1}H can be observed for a peptide in membranes suspended in H_2O by the use of both ^{1}H MAS and water suppression. It may also be remarked that baseline distortions associated with the tails of the intense lipid peaks interfere with the examination of these low-field peaks. A method that removes such baseline distortions is described below.

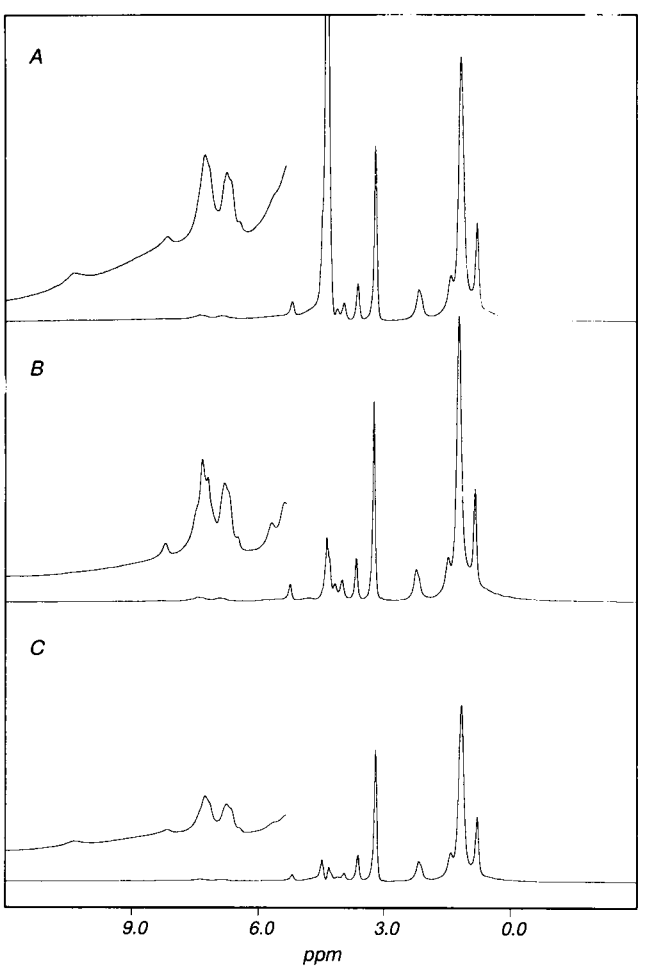

FIGURE 1 MAS ^{1}H NMR spectra of gramicidin/DLPC (mole ratio 1:20, lipid concentration 50% w/w) recorded at 50°C in H_2O (*A*) or in ^2H_2O (*B*) with single pulse excitation and in H_2O (*C*) with presaturation. A 65× expansion of the low-field region is shown above each spectrum. The intensities of spectra A and C are directly comparable.

The water suppression method is also efficient under more extreme conditions. This was demonstrated by comparing ^{1}H MAS spectra recorded in the absence or presence of presaturation for a sample identical to that of Fig. 1, except that it was purposely made four times more diluted in H_2O (i.e., 12.5% lipid by weight). Under such conditions, the lipid resonances were 15–60 times less intense than the water resonances in the absence of presaturation. Presaturation promoted an efficient suppression of the latter in spite of the higher water content, so that the residual water resonance reached an intensity similar to that of the choline β resonance (not shown).

Selective excitation of peptide resonances and suppression of lipid resonances

The above experiments show that both exchangeable and nonexchangeable amide proton resonances of gramicidin A occurring in the low-field region can be observed by high-speed ^{1}H MAS in the presence of nondeuterated lipids. However, when using standard 1D excitation, analysis of these resonances appears to be partially hindered by the baseline distortions that arise from the lipid signal. Even at the low lipid-to-peptide ratio used above, the lipid resonances are up to 50 times more intense than the peptide resonances. This is even more pronounced in Fig. 2 *A*, in which a DLPC/gramicidin A sample prepared at a more physiological lipid-to-protein mole ratio of 60 is used. Here

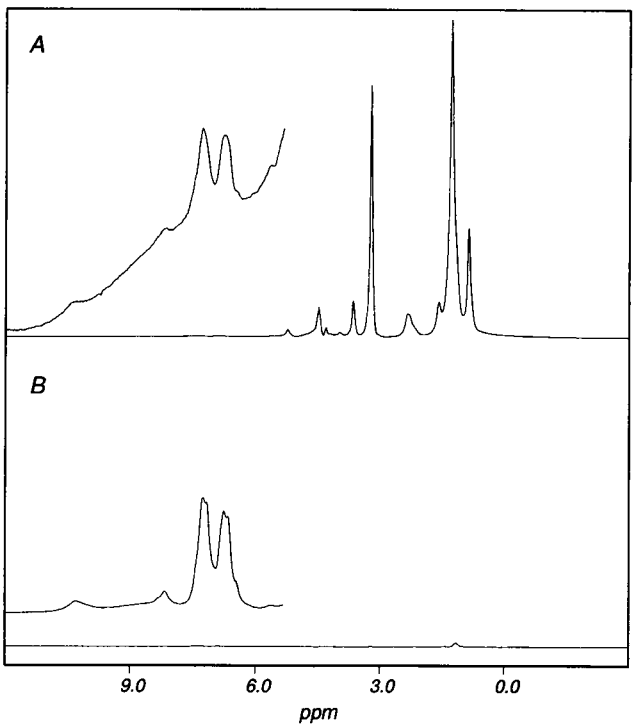

FIGURE 2 MAS ^{1}H NMR spectra of gramicidin/DLPC (mole ratio 1:60, lipid concentration 50% w/w) recorded at 50°C H_2O with presaturation (*A*) and with presaturation and regioselective excitation of the low-field peptide region (*B*). A 400× expansion of the low-field region is shown above each spectrum. The intensities of the two spectra are directly comparable.

baseline distortions that cannot be digitally corrected dramatically interfere with the observation of exchangeable and nonexchangeable peptide resonances. In a previous work (Seigneuret and Lévy, 1995) we have found a similar situation in solution NMR experiments on membrane peptides solubilized in nondeuterated detergents and shown that selective excitation of the low-field region can be used to overcome this difficulty. We have therefore attempted to apply this approach to ^{1}H MAS of membrane peptides. Fig. 2 *B* shows the spectrum of the same DLPC/gramicidin A sample (mole ratio 60) recorded with selective excitation of the low-field region. Selective excitation promotes a decrease of the lipid signal of up to 2 orders of magnitude. The low-field peptide resonances are little affected (less than 10% intensity decrease) and can now be observed without baseline distortion. Fig. 3, *A* and *B*, shows the low-field region of the samples used in Fig. 1 (DLPC/gramicidin A mole ratio of 20) in ^2H_2O and in H_2O with presaturation, respectively, both recorded with selective excitation. Because of the absence of baseline distortion, a better comparison of the peptide signals in H_2O and ^2H_2O can be made, allowing tentative assignments for these resonances. The most intense resonances situated between 6.5 and 7.5 ppm and the resonance at 8.29 ppm are stable in ^2H_2O and therefore are assigned, respectively, to aromatic (tryptophan H2-H7) and formyl ^{1}H, in agreement with Bouchard et al. (1995). The narrower linewidths of some resonances in

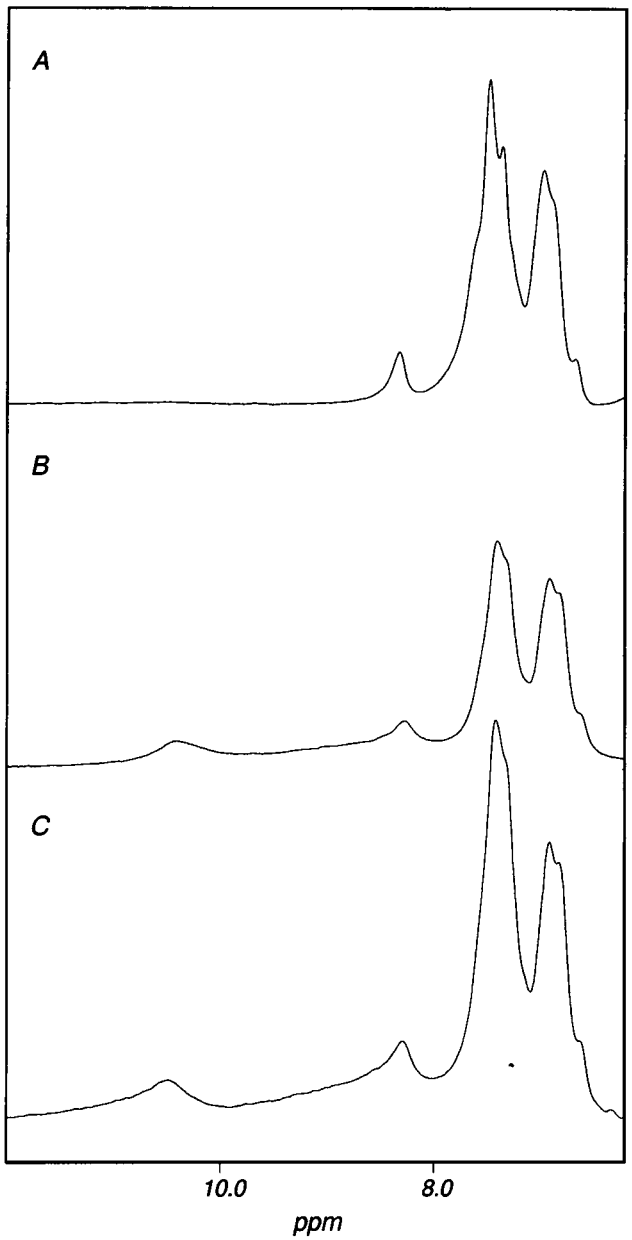

FIGURE 3 MAS ^{1}H NMR spectra of gramicidin/DLPC (mole ratio 1:20, lipid concentration 50% w/w) recorded at 50°C with regioselective excitation of the low-field peptide region in ^2H_2O (*A*), in H_2O with presaturation (*B*), and in H_2O with jump-return (*C*). The intensities of spectra B and C are directly comparable.

^2H_2O may be due to the decrease in dipolar interactions associated with peptide deuteration. The new resonances appearing in H_2O can also be assigned by comparison with the work of Arseniev et al. (1985) for gramicidin A in deuterated sodium dodecylsulfate. The resonance at 10.48 ppm in H_2O is attributable to exchangeable tryptophan indole ^{1}H (i.e., H1). Futhermore, the line at ~8.3 ppm appears to have a second component in H_2O due to a contribution from the ethylamide ^{1}H. In addition, because of the suppression of baseline distortions by selective excita-

tion, a broad, featureless line becomes apparent in H_2O between 8 and 9.5 ppm that was virtually undetectable in nonselective spectra. This originates from the unresolved amide ^1H resonances of gramicidin A. This indicates that both exchangeable and nonexchangeable ^1H of a membrane peptide in bilayer can be observed by ^1H MAS without any interference from nondeuterated lipids by using selective excitation and water suppression.

Comparison of water suppression methods

We have compared the application of two different water suppression schemes to MAS ^1H NMR of membrane peptides in bilayers, namely selective irradiation and jump-return (Plateau and Guéron, 1992). These two methods are intrinsically different, inasmuch as selective irradiation abolishes the solvent signal by saturating water ^1H, whereas jump-return achieves a selective nonexcitation of these protons. Both methods were tested on several bilayer-bound peptides and achieved satisfactory water suppression. Although as is also the case in solution NMR, jump-return was less efficient than presaturation, and the residual water peak became negligibly small in both cases, even for dilute membranes (not shown) if selective excitation of the low-field region was used concomitantly. However, significant differences were found between the two methods that were also dependent upon the peptide. Fig. 3 *C* shows the application of jump-return to DLPC/gramicidin A (20:1). Comparison with the spectrum obtained with presaturation (Fig. 3 *B*) indicates similar lineshapes in the amide/aromatic region. The lower intensity obtained with presaturation is due to a nonspecific effect already mentioned above for lipid resonances.

More profound differences between presaturation and jump-return water suppression were obtained for more superficially bound membrane peptides such as leucine-enkephalin (YGGFL). The latter peptide, although water-soluble, is known to bind to membranes and to penetrate partially into the bilayer (Behnam and Deber, 1984; Picone et al., 1990; Milon et al., 1990). ^1H MAS spectra of leucine-enkephalin incorporated into DLPC (lipid/peptide ratio 20:1) display a much higher resolution than for gramicidin A, and amide and aromatic resonances can be distinguished by comparison of spectra recorded in H_2O and ^2H_2O (see below). At 30°C the spectrum obtained with presaturation, apart from the nonspecific intensity decrease already mentioned, was found to display much more severely reduced intensities for amide resonances as compared to the jump-return spectrum (not shown). Such a phenomenon has been several times reported in solution ^1H NMR (Guéron et al., 1991) and arises from saturation transfer from water protons to amide protons due to the occurrence of significant although slow exchange between these two types of protons ($\tau_{ech} < 1$ s). Even with jump-return excitation, the ability to observe all resonances for such superficially bound peptides appears to depend on temperature. Indeed, with leucine-enkephalin, one amide resonance was found to vanish with a jump-return at 50°C (not shown). This suggests that, at this temperature, the exchange of this specific amide ^1H with water reaches the intermediate time scale ($\tau_{ech} \sim 1$ ms).

Spectral resolution in the amide-aromatic region of membrane peptides

The experiments shown above demonstrate that it is possible to selectively observe both exchangeable and nonexchangeable low-field protons of membrane peptides in nondeuterated lipid bilayers by ^1H high-speed MAS combined with presaturation and regioselective excitation. To assess the spectral resolution currently attainable in ^1H MAS in this low-field peptide region, we have recorded such ^1H spectra for various representative membrane peptides incorporated into bilayers. Fig. 4 compares the ^1H-MAS spectra low-field region of the intrinsic peptide gramicidin A, the hydrophobic peptide zfFG (Yeagle et al., 1993), the extrin-

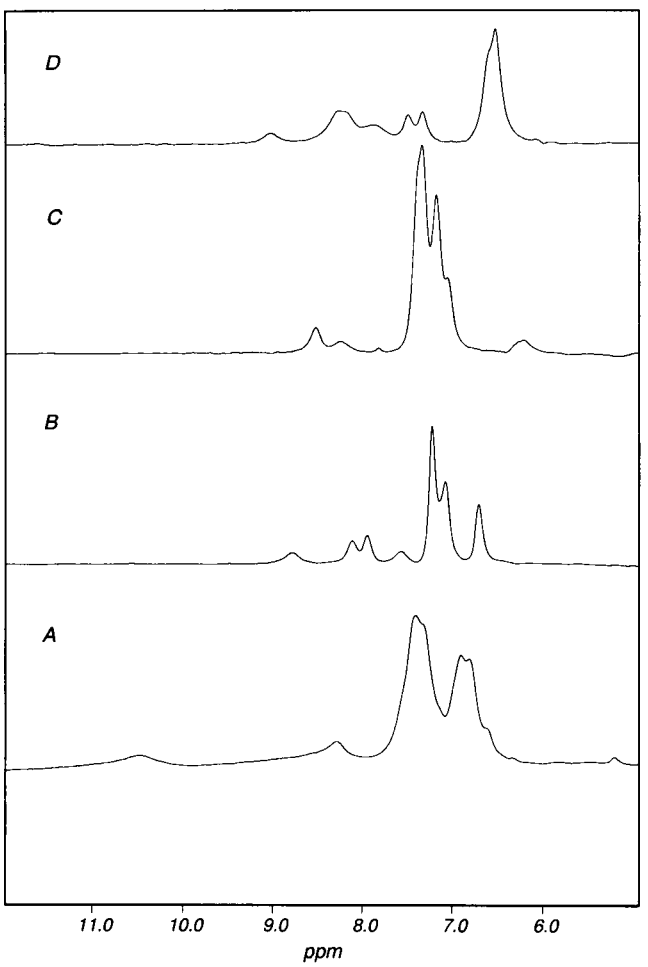

FIGURE 4 MAS ^1H NMR spectra of (*A*) gramicidin A/DLPC at 50°C, (*B*) leucine enkephalin/DLPC at 30°C, (*C*) zfFG/DMPC at 50°C, and (*D*) myr-KRTLR/DMPC at 40°C (mole ratio 1:20, lipid concentration 50% w/w) in H_2O recorded with regioselective excitation of the low-field peptide region and jump-return.

sic peptide Leu-enkephalin, and the lipopeptide MyrRK-TLR (Mosior and McLaughlin, 1991). All spectra were recorded in nondeuterated DLPC or DMPC and H_2O, using water suppression by jump-return and selective excitation of the amide-aromatic region. Gramicidin A, the largest peptide used (15 amino acids), yields largely unresolved amide resonances and only partially resolved aromatic resonances. On the other hand, the three other shorter peptides yield spectra for which many resonances originating from singly resolved ^1H can be observed. Tentative assignments for these resonances based on chemical shifts and comparison of spectra in H_2O and ^2H_2O are given in Table 1. Interestingly enough, the case of the lipopeptide indicates that not only amide and aromatic ^1H, but also exchangeable ^1H from arginine and lysine side chains are observable.

Regioselective 2D NOESY MAS ^1H NMR of peptides in nondeuterated lipid bilayers and H_2O

The results shown above indicate that, for small membrane peptides in bilayers, relatively well-resolved ^1H MAS NMR spectra can be obtained in H_2O, in the region corresponding to aromatic and amide protons. For a larger peptide, a partial resolution of some aromatic residues is obtained. To evaluate the significance of these results for structural studies, we have therefore attempted to extend this approach to multidimensional experiments. We have previously shown that regioselective solution 2D (Seigneuret and Lévy, 1995) and 3D (Le Guernevé and Seigneuret, in press) homonuclear ^1H spectra bearing most of the information contained in classical spectra can be obtained for membrane peptides solubilized in nondeuterated detergents. Here we have applied similar methods to solid-state ^1H MAS of membrane peptides in nondeuterated lipid multilayers suspended in H_2O. Whereas Bouchard et al. (1995) have reported a nonselective solid-state correlation spectroscopy experiment (COSY), we have examined the potential of regioselective NOESY experiments.

The interference of the resonances of protonated lipids was even stronger in 2D spectra than in 1D spectra. This is illustrated in Fig. 5, in which are compared two ^1H MAS NOESY spectra of DLPC/gramicidin A (mole ratio 20) recorded either classically or with regioselective excitation in the F2 dimension. Both spectra were recorded in H_2O with presaturation. In the nonselective experiment, the aliphatic region is dominated by lipid resonances, and in addition, the region correlating amide-aromatic ^1H with aliphatic ^1H is plagued with baseline distortions and ridges due to the edges of these lipid peaks. These artifacts can only very partially be corrected digitally and largely preclude an analysis of this region. Only the aromatic region appears to be exploitable. On the other hand, in the F2-regioselective NOESY experiment, because of the strong reduction of the lipid diagonal, the ridges are strongly reduced and can be removed by baseline correction (see below). In all, half of the spectral range that would be obtained with deuterated lipids becomes available.

Fig. 6 shows the low-field part of three F2-regioselective NOESY spectra of DLPC/gramicidin A (mole ratio 20) in H_2O recorded at three different mixing times. A jump-return was used for water suppression, and a local baseline correction was applied. The corresponding 1D spectrum of the sample is displayed on the side of the figure. All cross-peaks are positive (i.e., negative NOEs). At all mixing times, it appears that in the F2 dimension, only formyl and aromatic ^1H have cross-peaks with other ^1H. No cross-peaks originating from amide ^1H are observed. Tryptophan and formyl ^1H yield both intramolecular cross-peaks with other peptide ^1H and intermolecular cross-peaks with lipid ^1H. Cross-peaks with water also occur, originating from both ^1H exchange and cross-relaxation (see Otting et al., 1991; Volke and Pampel, 1995; Chen et al., 1996). The intrapeptide cross-peaks are more visible at the lower mixing times. In particular, several cross-peaks can be resolved within aromatic ^1H as well as between aromatic ^1H and aliphatic ^1H, presumably α, β, and γ ^1H. These arise from intraresidue or interresidue proximities. The intermolecular cross-peaks between peptide and lipids tend to dominate the spectra at high mixing times. These are due to proximities between peptide groups and lipid groups in direct contact. Current knowledge of the gramicidin A pore structure in the membrane environment (Arseniev et al., 1985; Ketchem et al., 1993) can be used to evaluate the spatial selectivity of these intermolecular NOESY connectivities. The four gramicidin A tryptophans are known to be located near the extremities of the pore, so that the corresponding ^1H are expected to yield cross-peaks mainly with ^1H from the lipid headgroup, glycerol backbone, and some methylene seg-

TABLE 1 Tentative assignments of the low-field ^1H resonances of membrane peptides in H_2O

Peptide	Chemical Shift (ppm)*		
	Amide	Aromatic	Other side chains
Gramicidin A	8.0–9.5 (14)	$Trp^{9,11,13,15}$: H_{2-7}: 7.36, 6.83, 6.62 (20) H_1: 10.48 (4)	Formyl: 8.29 (1) Ethylamido: ~8.4 (1)
Leucine-enkephalin	7.55 (1); 7.94 (1); 8.11 (1); 8.77 (1)	Tyr^1 H_{2-6}; Phe^4 H_{2-6}: 6.69, 7.06, 7.23 (9)	
zfFG	8.50 (2); 8.24 (1)	D-Phe^1 H_{2-6}; Phe^2 H_{2-6}, Cbz: 7.34; 7.16; 7.04 (15)	
Myr-KRTLR	9.03 (1); 8.23 (3); 7.87 (2)		Lys^1 H_ζ: 7.87 (1) $Arg^{2,5}$ H_ϵ: 7.48; 7.33 (2) H_ζ: 6.52 (6)

*The numbers in parentheses indicate the number of ^1H associated with each resonance or group of resonances.

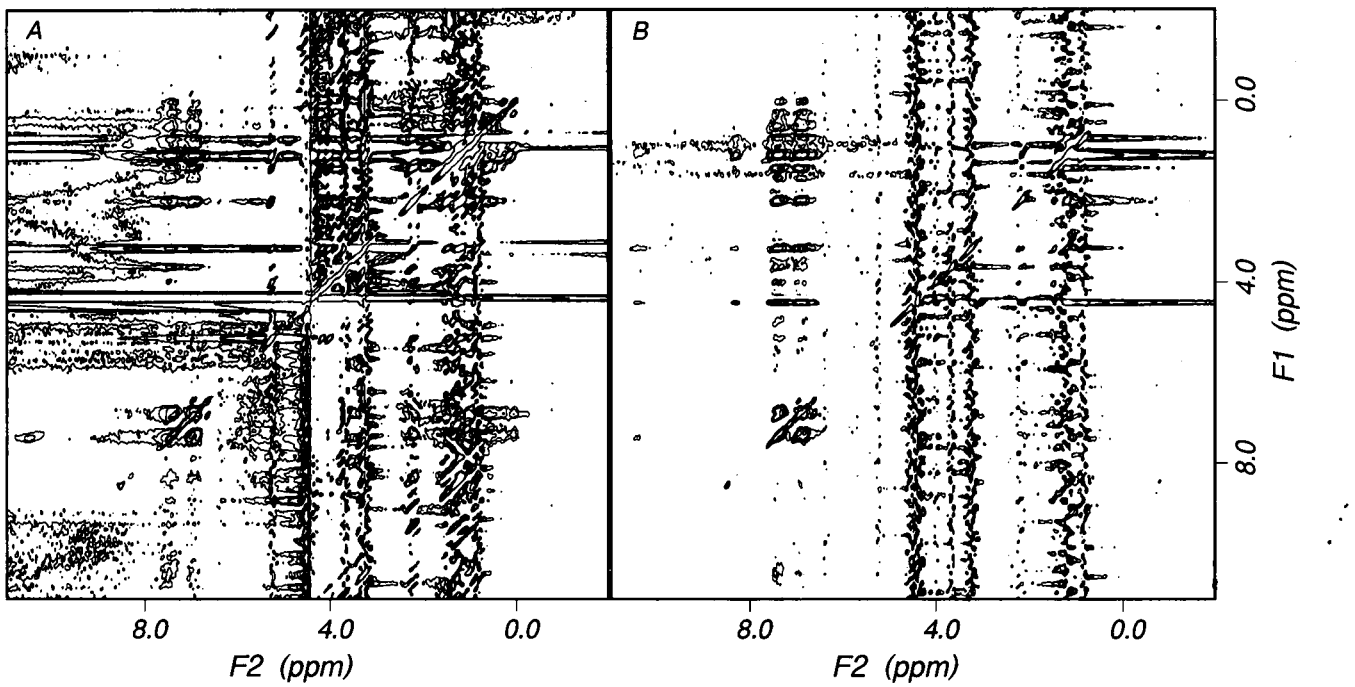

FIGURE 5 MAS ^{1}H 2D NOESY spectra of gramicidin/DLPC (mole ratio 1:20, lipid concentration 50% w/w) recorded at 50°C in H_2O with presaturation with (*A*) classical and (*B*) F2-regioselective excitation. The mixing time is 200 ms.

ments, as is indeed observed. On the other hand, the formyl moiety is expected to reside in the center of the bilayer, in agreement with the fact that cross-peaks mainly occur between its ^{1}H and terminal methyl and methylene lipid ^{1}H. These observations suggest that the spatial selectivity of the intermolecular NOESY is acceptable. Only at the highest mixing time are small features observed that could be attributed to spin diffusion, namely, cross-peaks between tryptophan and terminal methyl ^{1}H or between formyl and headgroup or glycerol ^{1}H.

A rather different situation is obtained in ^{1}H MAS F2-regioselective NOESY spectra of leucine-enkephalin in DLPC recorded in H_2O with a mixing time of 400 ms (Fig. 7). Here both amide and aromatic ^{1}H appear to yield visible cross-peaks. Spectra at lower mixing times had lower cross-peak intensities but were qualitatively similar. Furthermore, even at high mixing times, most of these cross-peaks occur at F1 frequencies that are distinct from those of lipid ^{1}H, suggesting that here, intramolecular NOESY connectivities within peptide ^{1}H are dominant. Well-resolved cross-peaks can be detected between amide ^{1}H, between amide and aromatic ^{1}H, and between amide-aromatic ^{1}H and aliphatic ^{1}H. In all, 24 cross-peaks unambiguously attributable to intrapeptide proximities are observable. Although determination of the peptide conformation from such data is beyond the scope of the present work, it may be remarked that the presence of only a few amide-amide and of numerous amide-α proximities is compatible with previous solution NMR work that suggested a turn conformation for leucine-enkephalin in a membrane-mimetic environment (Behnam and Deber, 1984; Picone et al., 1990; Milon et al., 1990). At high mixing times, a limited number of cross-peaks are observable with the lipids, principally between aromatic ^{1}H and methylene ^{1}H.

Regioselective 3D NOESY-NOESY MAS ^{1}H NMR of peptides in nondeuterated lipid bilayers and H_2O

Although, as demonstrated above, F2 regioselective excitation can be used to obtain 2D NOESY spectra of membrane peptides in nondeuterated lipid membranes, a limitation is that through-space correlations within aliphatic ^{1}H are not recovered by this approach. Again inspired by our previous work in solution NMR (Le Guernevé and Seigneuret, in press), we show here that 3D NOESY-NOESY experiments recorded with selective excitation in the acquisition dimension can be used to obtain such information. As illustrated in Fig. 8, NOESY correlations between two aliphatic ^{1}H can be detected in F3-regioselective NOESY-NOESY experiments, provided that one of these two aliphatic ^{1}H also yields a NOESY correlation with an amide or aromatic ^{1}H. An F3-regioselective 3D NOESY-NOESY spectrum of leucine-enkephalin in nondeuterated DLPC suspended in H_2O could be recorded in the reasonable time of 2 days. As in 2D experiments, a straightforward suppression of the lipid diagonal is obtained by regioselective excitation, allowing the observation of 50% of the information contained in a normal 3D cube. As an illustration of the potential usefulness of this experiment, a F1-F2 plane at the F3 frequency of 7.60 ppm corresponding to one of the amide

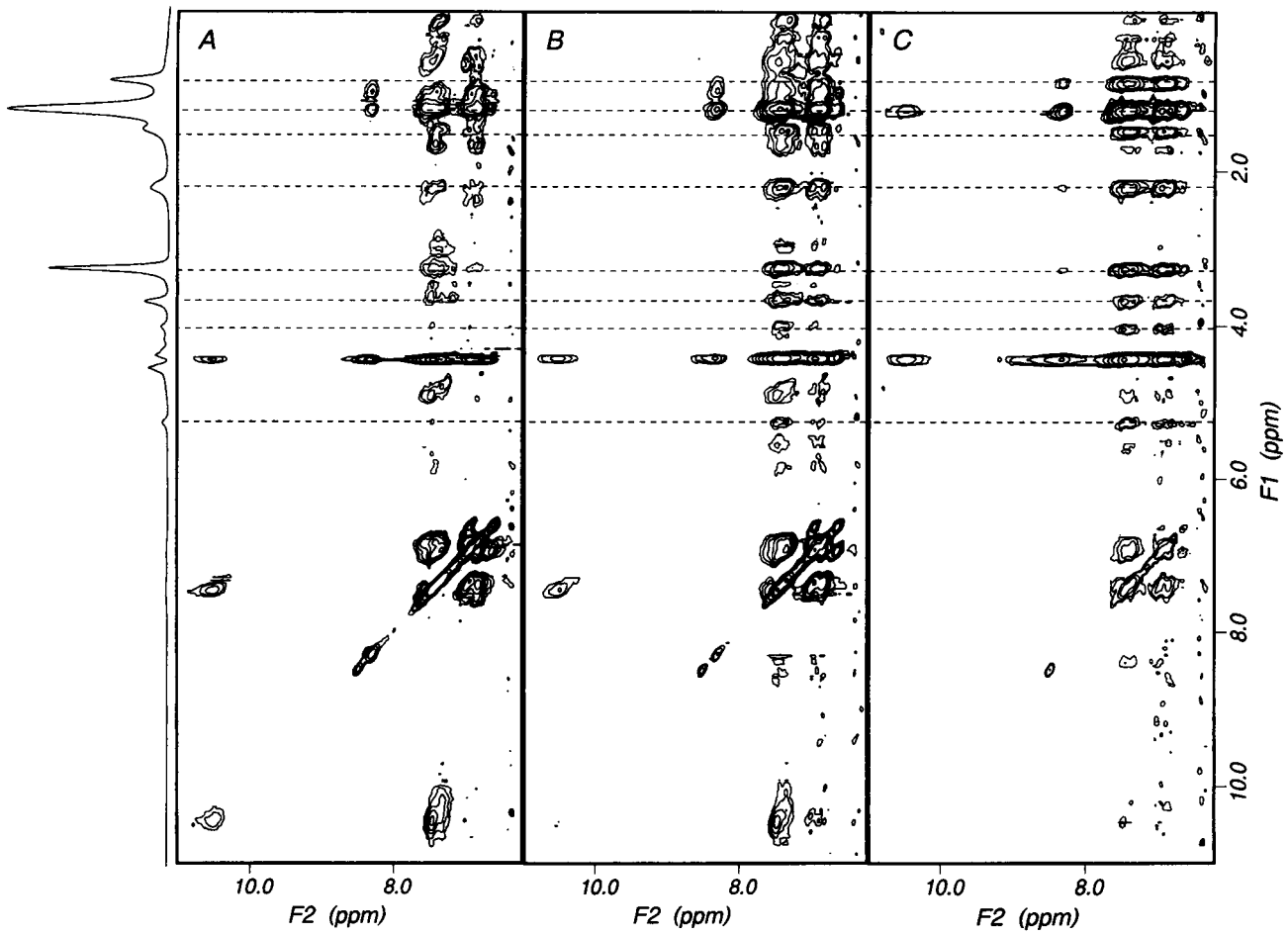

FIGURE 6 Low-field region of MAS ^{1}H F2-regioselective 2D NOESY spectra of gramicidin/DLPC (mole ratio 1:20, lipid concentration 50% w/w) recorded at 50°C in H_2O with jump-return at mixing times of (*A*) 30, (*B*) 100, and (*C*) 400 ms. The corresponding nonselective 1D spectrum is displayed on the left for comparison. The dotted lines correspond to the chemical shifts of the main lipid resonances.

^{1}H is displayed in Fig. 9. A detailed description of the analysis of such planes can be found in Boelens et al. (1989). The "cross-diagonal" peaks situated along the two lines marked N correspond to single NOESY transfers occurring during only one of the two mixing times. Accordingly, these are identical to the cross-peaks found in the 2D NOESY of Fig. 7 at the F2 frequency of 7.60 ppm. On the other hand, cross-peaks outside these lines correspond to double NOESY transfers. In particular, those cross-peaks in the upper left region of the plane are due to aliphatic → aliphatic → amide transfers. This demonstrates that through-space connectivities involving two aliphatic ^{1}H can be obtained by such regioselective 3D experiments in the presence of nondeuterated lipids.

DISCUSSION

The recent studies by Davis et al. (1995) and Bouchard et al. (1995) have demonstrated that it is possible to obtain ^{1}H spectra of membrane peptides in lipid bilayers with relatively narrow linewidths by using high-speed magic angle spinning. These investigations potentially open new possibilities in that they suggest that ^{1}H multidimensional approaches similar to those employed in solution NMR of soluble proteins might become applicable to membrane peptides in situ. The present study was aimed at introducing further developments of this method, to contribute to reaching this goal.

In this instance, one development is that brought by the possibility of observing resonances of exchangeable protons of membrane peptides by performing ^{1}H-MAS experiments in H_2O with the help of water suppression methods. A recent communication (Warschawski and Devaux, 1995) has shown the use of water suppression in MAS of phospholipids for which no exchangeable ^{1}H occur. Here we have shown that both backbone and side-chain exchangeable ^{1}H can be observed after water suppression, even for relatively dilute samples. Provided that jump-return is used and that appropriate temperature and pH are chosen, even ^{1}H with relatively fast exchange rates seem to be observable. The importance of being able to study peptide amide ^{1}H is twofold. First, because of the strong sensitivity of amide ^{1}H chemical shift to local conformation, sequence position, and environment, a relatively wide resonance dis-

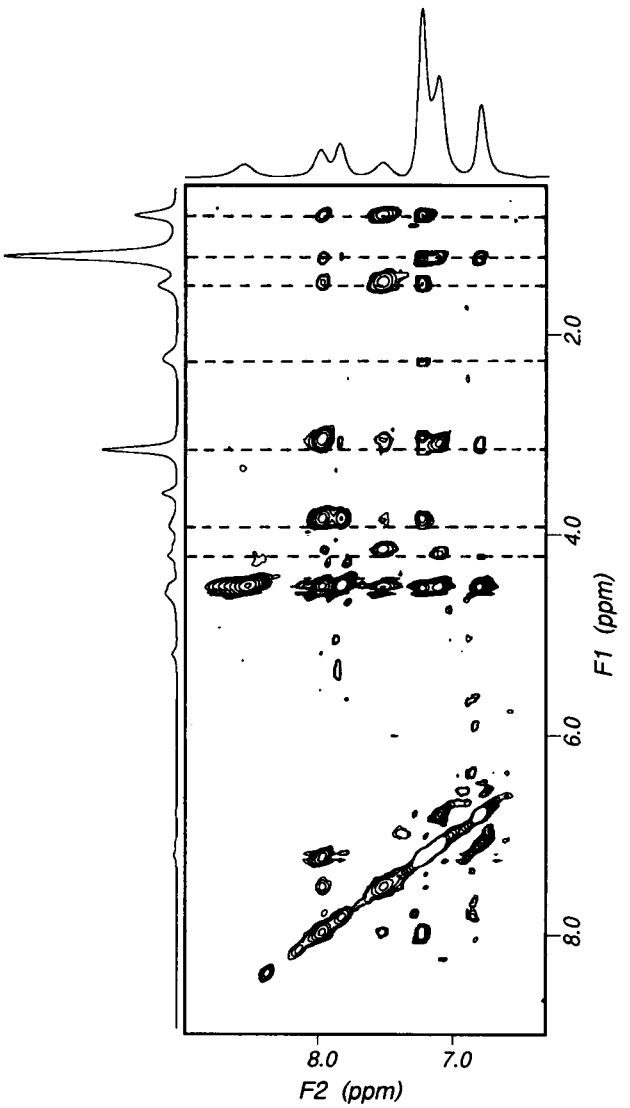

FIGURE 7 Low-field region of the MAS ^{1}H F2-regioselective 2D NOESY spectrum of leucine enkephalin/DLPC (mole ratio 1:20, lipid concentration 50% w/w) recorded at 30°C in H_2O with jump-return at a mixing time of 400 ms. The corresponding nonselective 1D spectrum is displayed on the left, and the regioselective 1D spectrum is displayed on the top for comparison. The dotted lines correspond to the chemical shifts of the main lipid resonances.

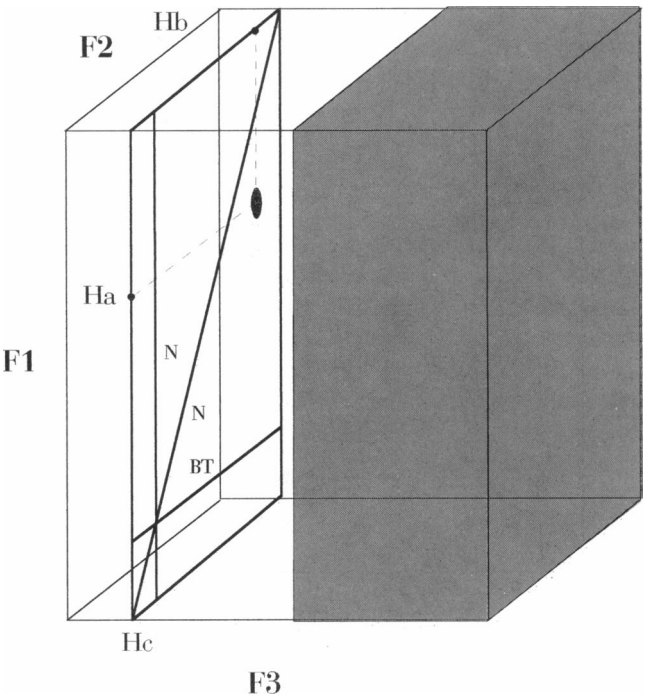

FIGURE 8 Principle of the observation of correlations between aliphatic ^{1}H on the F3-regioselective MAS 3D NOESY-NOESY spectrum of a membrane peptide in nondeuterated lipid membranes. The grayed portion of the 3D cube is the nonselected region. Double NOESY transfers between three protons Ha (aliphatic), Hb (aliphatic), and Hc (amide or aromatic) yield cross-peaks that are best viewed in the F1-F2 plane corresponding to the F3 frequency of the Hc proton. One such plane is emphasized on the figure, and the two NOESY lines (N) and the back-transfer line (BT) are indicated.

persion is usually observed in this region as compared to other peptide proton types. Therefore a higher resolution in ^{1}H MAS spectra of membrane peptide may be expected by observing such amide ^{1}H, provided that sufficiently narrow linewidths are reached for individual resonances. This is important for structural studies that mainly rely on being able to identify single ^{1}H resonances. Moreover, amide ^{1}H resonances are key features for polypeptide conformational studies by NMR. The experience gained from solution NMR indicates that determination of peptide and protein secondary structure is mainly based on the measurement of through-space connectivities involving amide ^{1}H (e.g., NH-NH or C_αH-NH; see Wüthrich, 1986). Besides amide ^{1}H, the ability to observe other exchangeable ^{1}H from Trp, Lys, Arg, and possibly other side chains is also notable, because these are often involved in specific interactions at the membrane surface.

However, our experiments also indicate that, at least at a 400-MHz frequency, the resolution attainable for such exchangeable ^{1}H, and specially for amide ^{1}H, strongly depends on the specific membrane peptide studied. Whereas for small amphiphilic or hydrophobic oligopeptides, complete or nearly complete resolution of amide residues is obtained in H_2O, for the intrinsic 15-residue peptide gramicidin A, amide resonances, although clearly visible, are displayed as a broad, featureless region. Such a low resolution also contrasts with the narrower linewidths of 40–80 Hz obtained both by Bouchard et al. (1995) and by us for gramicidin A aromatic and formyl ^{1}H, as well as by Davis et al. (1995) for aliphatic ^{1}H of another transmembrane peptide. The lack of resolution obtained by MAS in the gramicidin amide ^{1}H region may come in part from the superposition of 14 distinct resonances within a ~1.5 ppm range. However, this probably cannot account for the complete lack of resolution, because we have found that a synthetic spectrum generated from the ^{1}H amide chemical shifts of Arseniev et al. (1985) and using individual line-

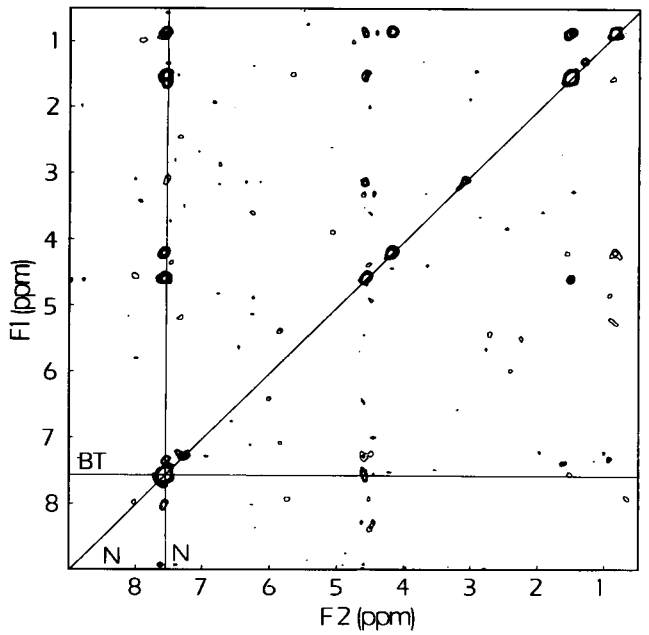

FIGURE 9 F1-F2 plane corresponding to an F3 frequency of 7.60 ppm of the MAS ^{1}H 3D NOESY-NOESY spectrum of leucine enkephalin/DLPC (mole ratio 1:20, lipid concentration 50% w/w) recorded at 30°C in H_2O with jump-return using two mixing times of 200 ms. The two NOESY lines (N) and the back-transfer line (BT) are indicated.

widths of 80 Hz displays a partially resolved structure (not shown). Furthermore, it may also be remarked that, in spite of the higher resolution expected from a 2D experiment, none of the NH-C_αH cross-peaks associated with the $\beta^{6,3}$-helical structure (Arseniev et al., 1985) is observed in MAS-NOESY experiments. This suggests that amide (and possibly C_αH) resonances may have intrinsically broader linewidths than aliphatic and aromatic side-chain resonances in MAS spectra of transmembrane peptides. This can be explained using the formalism developed by Davis et al. (1995; see also Sekine et al., 1990). These authors indicated that the residual ^{1}H linewidth of a membrane peptide resonance under MAS was determined by the modulation of the residual dipolar interaction (expressed as its second moment ΔM_2) by intermediate-scale motion due to, e.g., rigid body wobbling. We suggest that ΔM_2 may be determined by motional averaging due to rapid axial rotation of the peptide, as considered by Davis et al. (1995), but also to rapid local librational or torsional motion of peptide groups. This latter motion is likely to be significantly different for amide ^{1}H and side-chain ^{1}H. Recent ^{2}H and ^{15}N solid-state NMR studies on gramicidin A local dynamics indicate that such motions occur in the nanosecond range. Furthermore, amide groups appear to undergo only low-amplitude librations (~5° rms; North and Cross, 1995) in comparison with those displayed by tryptophan side chains (~25° rms; Hu et al., 1995) and to the relatively ample torsional motion of aliphatic side chains (Killian et al., 1992; Lee and Cross, 1994). Thus a higher ΔM_2 and therefore larger linewidths are expected for amide ^{1}H than for side-chain ^{1}H for intrinsic peptides. The narrower amide linewidths obtained for the oligopeptides leucine-enkephalin and zfFG as well as for the lipopeptide myr-KRTLR can be explained by larger librational amplitudes due to a more flexible backbone and/or by a higher rate of the rigid-body wobbling motion.

A second development carried out in this study is the use of regioselective excitation that provides the ability to observe most membrane peptide ^{1}H resonances in ^{1}H MAS spectra in the presence of nondeuterated lipids, even at a relatively high lipid-to-peptide ratio. It must be stressed that the selective excitation scheme used in these experiments is easy to implement and does not require sophisticated NMR hardware (Roumestand et al., 1995). Low-field amide and aromatic resonances of peptides can be directly observed on 1D spectra using such regioselective excitation to remove the lipid signal. Furthermore, in favorable cases, high-field peptide aliphatic resonances that are hidden by the lipids in 1D spectra can be observed through their 2D connectivities to amide and aromatic ^{1}H in F2-regioselective NOESY spectra. Peptide aliphatic-aliphatic connectivities can also be obtained from regioselective 3D NOESY-NOESY. Previous authors have used different strategies to solve this peptide-lipid overlap problem. Davis et al. (1995) used deuterated lipids to fully observe the peptide spectrum. Although this approach is certainly the most efficient, it requires that the chosen lipids be available or synthesizable in deuterated form. The binding of a particular peptide to membranes, achievement of the required conformation, and optimal spectral resolution may require specific lipids. Furthermore, as suggested here, the use of nondeuterated lipids may provide new information on lipid-peptide interactions and peptide topography. Bouchard et al. (1995) were able to observe aromatic resonances of gramicidin A in nondeuterated DMPC using standard 1D and 2D spectra. It seems that this approach is limited to low lipid-to-protein ratios and to specific regions of 2D spectra.

By the use of both water suppression and lipid suppression schemes, it was possible to obtain 1D and multidimensional ^{1}H spectra of several membrane peptides. One-dimensional experiments can be used to assess the membrane topography of the peptides by the use of paramagnetic agents (Papavoine et al., 1994) as well as to study peptide dynamics through relaxation measurements. On the other hand, multidimensional experiments are expected to allow a direct study of peptide structure and lipid-protein interactions through measurements of intramolecular and intermolecular dipolar connectivities. Our experiments indicate, however, that the information currently avaible from these experiments depends to a large extent on the studied peptide. In the case of leucine-enkephalin, 2D NOESY and 3D NOESY-NOESY spectra displaying extensive intrapeptide ^{1}H connectivities could be obtained. The number of such connectivities is comparable to that used in previous solution NMR studies of the conformation of this peptide (Picone et al., 1990; Milon et al., 1990). This suggests that structure determination from MAS ^{1}H NMR data is potentially possible for small peptides bound to membranes. The

next step toward this goal is the design of systematic spectroscopic and computational strategies. Unlike in solution NMR, the concerted use of both scalar and dipolar interproton correlations, which allows sequence-specific assignments and structure determination to be performed sequentially, is not possible in MAS ^{1}H NMR of membrane peptides. However, it has been shown that structure determination from NOESY data without the prior need of assignments is possible in particular cases (Malliavin et al., 1992). Such an approach may be adapted to ^{1}H MAS NOESY data. In the case of transmembrane peptides such as gramicidin A, our results suggest that backbone ^{1}H resonances are currently too broad at 400 MHz to allow extensive structural determination from NOESY spectra. This may not hold at higher field strengths. In addition, Davis et al. (1995) have suggested several other approaches by which the ^{1}H MAS membrane peptide linewidths could be decreased, including the use of higher spinning speeds. Even in the current situation, measurement of specific distances between aliphatic or aromatic side chain ^{1}H of transmembrane peptides may be possible, because these appear to yield narrower resonances. Furthermore, we have also shown that NOESY connectivities can be measured between specific lipid ^{1}H and transmembrane peptide ^{1}H. Possible applications of such information include the study of lipid-peptide interaction and their specificity and of peptide membrane topography and lipid-exposed surface, as well as the evaluation of results from molecular dynamics simulation (e.g. Woolf and Roux, 1996). A related approach has been proposed by Smith et al. (1994) using ^{13}C labeling and rotational resonance. A potential problem in the use of NOESY data to assess intrapeptide or lipid-peptide proximities is the occurrence of spin diffusion. In this regard, our results agree with those of Forbes et al. (1988a), which indicate that spin diffusion does occur in membrane NOESY experiments but is of limited efficiency. Specific approaches have been designed in solution to account for spin diffusion that could be adapted to ^{1}H MAS, including the use of rotating-frame NOESY (Bax et al., 1986) and 3D NOESY-NOESY (Boelens et al., 1989).

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