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Determination of Membrane Peptide Orientation by ^{1}H-Detected ^{2}H NMR Spectroscopy

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We demonstrate the application of the *pr*oton *i*nverse detected *de*uteron (PRIDE) NMR technique to the measurement of the orientation of membrane-bound peptides with enhanced sensitivity. Gramicidin D, a transmembrane peptide, and ovispirin, a surface-bound peptide, were used as model systems. The peptides were ^{2}H-labeled by ^{1}H/^{2}H exchange and oriented uniaxially on glass plates. The directly detected ^{2}H spectra of both peptides showed only a strong D_2O signal and no large quadrupolar splittings. In contrast, the PRIDE spectrum of gramicidin exhibited quadrupolar splittings as large as 281 kHz, consistent with its transmembrane orientation. Moreover, the large D_2O signal in the directly detected ^{2}H spectra was cleanly suppressed in the PRIDE spectrum. For ovispirin, the ^{1}H indirectly detected ^{2}H spectrum revealed a 104 kHz splitting and a zero-frequency peak. The former reflects the in-plane orientation of most of the helix axis, while the latter results from residues with a magic-angle orientation of the N–D bonds. These are consistent with previous ^{15}N NMR results on ovispirin. The combination of PRIDE and exchange labeling provides an economical and sensitive method of studying membrane peptide orientations in lipid bilayers without the influence of D_2O and with the ability to detect N–D bonds at the magic angle from the bilayer normal. © 2002 Elsevier Science (USA)

Key Words: **^{2}H NMR; orientation; membrane peptide; PRIDE; sensitivity enhancement.**

INTRODUCTION

Membrane-associated peptides and proteins play an essential role in many biological processes such as signal transduction, membrane transport, and energy conversion. Knowledge of the three-dimensional structure of these molecules is critical for understanding their function. The standard approaches for structure determination, X-ray diffraction and solution NMR, are not always straightforward for studying these membrane systems because of difficulties in crystallization and solubilization. Solid-state NMR, on the other hand, provides a valuable alternative for studying these membrane peptides in their natural environment of lipid bilayers. High-resolution solid-state NMR spectra of membrane peptides can now be obtained by magic-angle spinning (*1–4*), macroscopic orientation (*5–10*), or the combination (*11*) (*12*).

However, the sensitivity of solid-state NMR spectroscopy of membrane peptides remains a challenge, since the amount of sample available is typically limited, and usually a low-frequency nuclear spin species such as ^{15}N or ^{13}C is detected. The sensitivity can be enhanced using ^{1}H indirect detection of these low-frequency spins, by taking advantage of the high gyromagnetic ratio of the ^{1}H spins. This approach has recently been demonstrated for ^{15}N and ^{13}C spectroscopy, both under magic-angle spinning and under the static conditions (*13–15*).

Static ^{15}N NMR of uniaxially aligned helical membrane peptides has yielded rich information on the orientation of membrane peptides in lipid bilayers. Commonly, the ^{15}N chemical shift interaction and the ^{15}N–^{1}H dipolar coupling are used to probe the helix orientation (*16, 17*). But in principle, any spin interaction that is parallel to the helix axis can be used. The amide ^{2}H (D) quadrupolar coupling is such an interaction and indeed has been utilized (*18, 19*). In contrast to the ^{15}N experiments, which require ^{15}N labeled amino acids during the peptide synthesis, ^{2}H labeling of the amide hydrogens can be achieved for multiple residues simultaneously through chemical exchange in D_2O. This relatively simple and economical way of isotopic labeling provides a strong motivation for using ^{2}H NMR to measure the orientation of membrane peptides in the lipid bilayer. Moreover, the large quadrupolar couplings of N–D and C–D groups (ca. 200–300 kHz) make them sensitive probes of segmental orientation as well as segmental motion (*20*) (*21*).

To fully realize the potential of ^{2}H NMR for studying membrane peptide orientations, we need to increase the sensitivity of the ^{2}H spectra. Recently, a *pr*oton *i*nverse detected *de*uteron (PRIDE) NMR technique suitable for non-spinning samples was developed (*22*). The method exploits the large difference in the ^{1}H and ^{2}H gyromagnetic ratios ($\gamma_H/\gamma_D = 6.49$) to gain sensitivity, and it makes use of pulsed spin–lock detection to focus all ^{1}H intensities to the zero frequency of the direct dimension (ω_2), thus further increasing the sensitivity of the ^{2}H dimension (ω_1). Using this approach, a 20-fold signal-to-noise (S/N) enhancement was achieved. The PRIDE pulse sequence used in this work is shown in Fig. 1 and is slightly modified from the original version (*22*). The ^{1}H and ^{2}H coherence transfer is accomplished by excitation of the heteronuclear multiple-quantum coherence (HMQC). Since this is based on the dipolar couplings between

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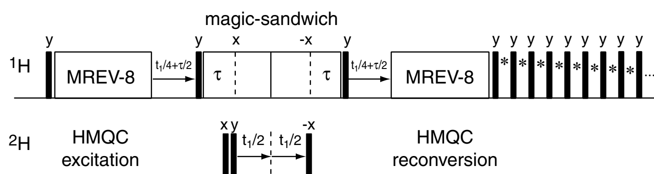

FIG. 1. Pulse sequence for the PRIDE experiment. The ^{1}H–^{2}H heteronuclear multiple quantum coherence (HMQC) is excited, evolves under the ^{2}H quadrupolar interaction during t_1, and is then refocused to ^{1}H single-quantum coherence for detection. The ^{1}H spectrum is detected under a pulsed-spin–lock sequence, which concentrates all ^{1}H intensities into a zero-frequency peak, further increasing the sensitivity gain. During the t_1 period, the ^{1}H–^{1}H homonuclear interaction is suppressed by a magic-sandwich sequence.

the ^{2}H and ^{1}H spins, isotropically mobile deuterons such as D_2O are suppressed in the PRIDE spectra. The ^{1}H–^{2}H HMQC evolves under the ^{2}H quadrupolar interaction during t_1, while the ^{1}H–^{1}H homonuclear interaction is removed by a magic-sandwich sequence (*23*).

In this paper, we demonstrate the application of the PRIDE technique to the study of the orientation of membrane peptides that are natural abundance in ^{15}N. Two peptides, gramicidin D, which is transmembrane, and ovispirin, which is parallel to the membrane surface, are used as model systems. These peptides are exchange-labeled with ^{2}H at low levels. We show that the PRIDE technique yields ^{2}H quadrupolar splittings consistent with the known orientations of gramicidin and ovispirin, while the direct ^{2}H detection did not yield any observable splittings due to the low ^{2}H labeling levels. Further, we show that the suppression of isotropically mobile deuterons is advantageous for observing peptide helices that are oriented at the magic angle from the bilayer normal.

RESULTS

Gramicidin is a β-helix with 6.5 residues per turn and with the helical axis parallel to the bilayer normal (*6, 24*). Figure 2 shows the ^{2}H spectra of 5 mg exchange-labeled gramicidin at a peptide : lipid molar ratio of 1 : 20. The sample was oriented with the bilayer normal parallel to the external magnetic field. The directly detected ^{2}H spectrum (Fig. 2a) after 7168 scans showed no ^{2}H signals except for the central D_2O peak. A 1000-fold vertical expansion (Fig. 2b) indicates more clearly the absence of quadrupolar splittings.

While the amounts of peptide and signal averaging were insufficient for observing any quadrupolar splittings through direct ^{2}H detection, the same number of deuterons yielded clearly observable splittings in the PRIDE spectrum (Fig. 2c) after only twice the number of scans. The spectrum corresponds to the $\omega_2 = 0$ cross section of the 2D spectrum. The S/N of the highest peak in the PRIDE spectrum is about 7.4. If the same number of scans as the direct detection experiment was used, then the S/N would be 5.2. The four quadrupolar splittings are 125, 203, 254, and 281 kHz. Based on previous ^{2}H experiments (*25, 26*), the smallest splitting can be assigned to Trp indole rings, the 203 and 254 kHz splittings result from the amide deuterons of even-numbered residues (D amino acids), and the 281 kHz splitting corresponds to the odd-numbered residues (L amino acids).

Since direct ^{2}H detection did not yield observable peaks, we estimated the maximum ^{2}H labeling level of gramicidin by comparing with chain-perdeuterated POPC. The ^{2}H spectra of a series of oriented POPC-d_{31} lipids with decreasing sample amounts were measured by direct detection (Fig. 3). The lipid

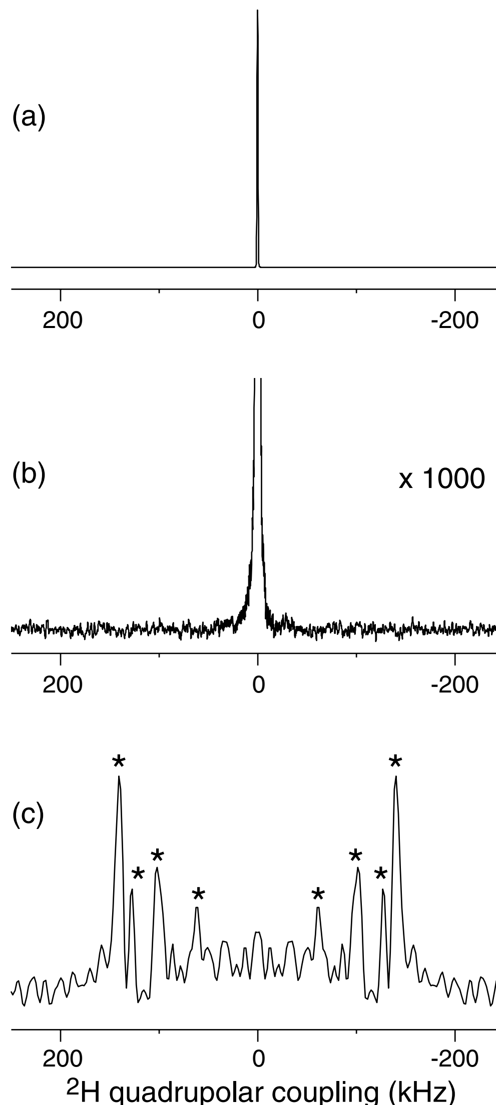

FIG. 2. ^{2}H spectra of 5 mg exchange-labeled gramicidin D, at a peptide : lipid molar ratio of 1 : 20. The bilayer normal is parallel to the magnetic field. (a) Directly detected ^{2}H spectrum. Number of scans (NS): 7168. The ^{2}H spectral width and time domain size were 1 MHz and 3072 points, respectively. (b) Spectrum (a) magnified 1000 times vertically. (c) ^{1}H indirectly detected ^{2}H spectrum (PRIDE). Stars indicate the observed splittings. The spectrum was acquired with 112 scans per t_1 slice and 128 t_1 slices. Both spectra were acquired at 295 K, above the phase transition temperature of the DLPC lipid.

amount varied from 4.46 to 0.073 mg, a 64-fold reduction. The ^{2}H spectra were collected under identical conditions to the gramicidin spectrum in Fig. 2a. It can be seen that the 0.073 mg POPC-d_{31} sample represents the sensitivity limit of direct detection (Fig. 3d). The number of ^{2}H spins in this lipid sample corresponds to a ^{2}H labeling level of 5% for gramicidin, taking into account all exchangeable sites in the peptide. In other words, at most 0.13 μmol of gramicidin molecules are ^{2}H exchange-labeled. While this number of spins is too low to yield any observable signal in the directly detected ^{2}H spectrum, it is still sufficient to give clear signals in the PRIDE ^{2}H spectrum. This demonstrates the sensitivity enhancement of the PRIDE technique.

Figure 4 shows the ^{2}H spectra of 2.5 mg exchange-labeled ovispirin at a peptide : lipid molar ratio of 1 : 27. The sample was oriented with the bilayer normal parallel to the magnetic field. The directly detected ^{2}H spectrum (Figs. 4a and 4b) was acquired after 6016 scans. Similar to gramicidin, we observed only a dominant D_2O signal at the center of the spectrum, and we did not detect any larger splittings. In contrast, the PRIDE spectrum of the same sample (Fig. 4c), averaged for 20480 scans, exhibited a quadrupolar splitting of 104 kHz and a zero-frequency peak of comparable height with a full-width-at-half-maximum of about 20 kHz. Using a quadrupolar coupling constant (e^2qQ/h) of 209 kHz, the 104 kHz splitting corresponds to an angle of about 70° between the backbone N–D bonds and the bilayer normal. The broad zero-frequency peak, on the other hand, corresponds to an orientation around the magic angle ($55° \pm 3°$) from the bilayer normal. The S/N of the ovispirin PRIDE spectrum is about 14. With the same number of scans as the directly detected experiment, the S/N would be about 7.5, which is still better than the directly detected spectrum in Fig. 4a.

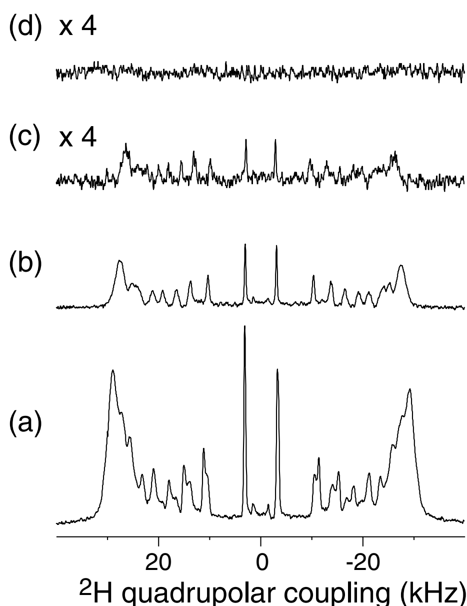

FIG. 3. Directly detected ^{2}H spectra of oriented POPC-d_{31}. Lipid amounts are (a) 4.46 mg, (b) 1.16 mg, (c) 0.29 mg, and (d) 0.073 mg. Each spectrum was acquired with 7168 scans at 293 K.

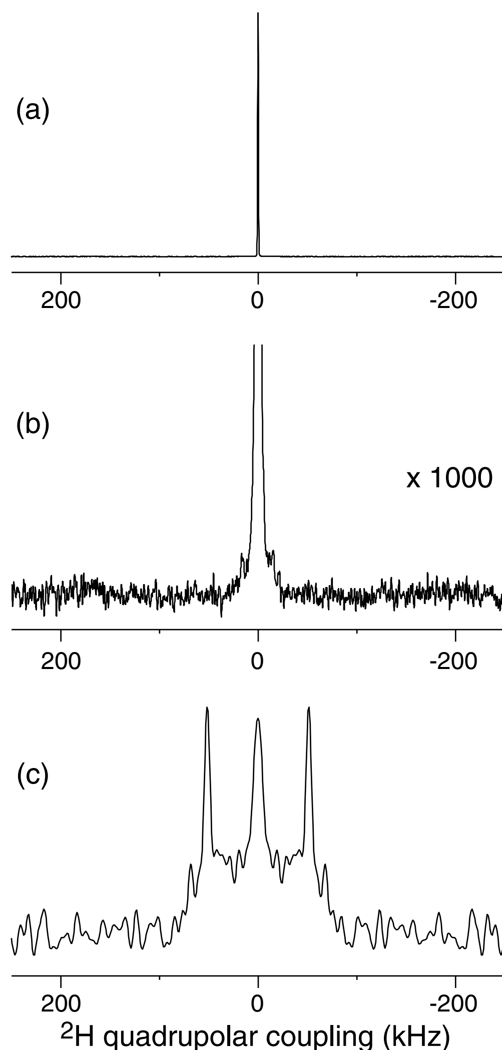

FIG. 4. ^{2}H spectra of 2.5 mg exchange-labeled ovispirin, with a peptide : lipid molar ratio of 1 : 27. (a) Directly detected ^{2}H spectrum. NS: 6016. ^{2}H sweep width: 500 kHz; (b) spectrum (a) magnified 1000 times vertically; (c) PRIDE spectrum on the same sample. The spectrum was acquired with 160 scans per t_1 slice and 128 t_1 slices. Both spectra were acquired at 294 K.

DISCUSSION

Static ^{2}H NMR is a powerful method for investigating the molecular dynamics and structure of organic compounds. Applied to membrane peptides, ^{2}H NMR can provide valuable orientation information, since the direction of the amide ^{2}H quadrupolar interaction reflects the orientation of helix axes or β-strand axes relative to the bilayer normal.

The PRIDE ^{2}H spectrum of gramicidin shown here is qualitatively consistent with previous directly detected ^{2}H spectra

(*25, 26*). A small increase in the linewidths and splittings is attributed to the temperature-dependence of gramicidin and lipid motions. Specifically, Prosser *et al.* resolved a dozen ^{2}H splittings at 65°C but only 8 splittings at 35°C, using the same lipid DLPC (*25*). Since the current PRIDE spectrum was acquired at a lower temperature of 22°C, where the order parameter of the lipids and the peptide is larger, the slightly increased quadrupolar splittings and broadened lines are to be expected. Note that the inherent resolution of the ^{2}H dimension of the PRIDE experiment is better than the resolution of directly detected ^{2}H spectra, since ^{1}H homonuclear decoupling is employed during ^{2}H evolution in the PRIDE experiment.

While gramicidin provides an example of a transmembrane peptide with large quadrupolar splittings of above 250 kHz, ovispirin represents a surface-bound peptide with smaller ^{2}H quadrupolar couplings. The lack of any significant splittings in the directly detected ^{2}H spectrum (Fig. 4a), contrasted with the distinct 104 kHz splitting in the PRIDE spectrum (Fig. 4c), again confirm the sensitivity enhancement due to ^{1}H indirect detection. To convert the detected splitting to an average N–D bond orientation, we assumed a quadrupolar coupling constant of 209 kHz and an asymmetry parameter (η) of 0. In reality, η of amide deuterons is usually around 0.18 (*19*) and the quadrupolar coupling constant is somewhat variable depending on the hydrogen bond length (*25*). These are sources of uncertainty in orientation determination by ^{2}H NMR in general. Similar levels of uncertainties are also present in orientation determination through ^{15}N NMR, due to variations in the ^{15}N chemical shift tensor orientation and magnitude. Nevertheless, the estimated 70° angle between the N–D bonds and the bilayer normal is roughly consistent with ^{15}N–^{1}H dipolar couplings of about 4 kHz measured for this peptide (*27*).

The zero-frequency signal in the PRIDE spectrum of ovispirin does not result from D_2O. Rather, they are primarily due to amide deuterons whose N–D bonds are tilted at the magic angle (54.7°) from the bilayer normal. In addition, there may be a small contribution from mobile side chains with exchangeable hydrogens. The exclusion of the isotropic water signal is based on the fact that the ^{1}H–^{2}H multiple-quantum coherence is excited through the dipolar coupling, which is averaged to zero for D_2O. Thus, only rigid and anisotropically mobile segments can be detected in the PRIDE spectra. The similarly D_2O-rich gramicidin sample showed no zero-frequency intensity in its PRIDE spectrum (Fig. 2c), thus verifying the suppression of isotropic signals. In comparison, side chain motions are usually more anisotropic, thus can contribute to the PRIDE spectra. Ovispirin contains Lys, Arg, Asn, Tyr, and His residues, which have exchangeable ND, ND_2, ND_3, and OD groups. These deuterons can contribute small unresolved splittings to the broad central signal in the spectrum, but probably at much lower intensities per deuteron due to the motional averaging.

The assignment of the significant zero-frequency signal mainly to N–D groups that are oriented near the magic angle to the bilayer normal is consistent with recent ^{15}N NMR measurements, which detected a near-zero ^{15}N–^{1}H dipolar coupling and an ^{15}N anisotropic chemical shift of 109 ppm, close to the isotropic shift of 107 ppm. These small anisotropies were definitively assigned to the N–H bond of Gly-18 (*27*). The zero-frequency signal in the PRIDE spectrum supports the ^{15}N NMR results of the existence of a distorted helix. But while the previous ^{15}N experiments only detected five labeled residues (Leu-3, Ile-6, Ile-11, Ile-13, and Gly-18), the current PRIDE experiment observes the entire peptide. The relative intensity of the zero-frequency peak and the splitting suggests that at most 20–30% of the residues in the peptide have a magic-angle N–D bond orientation from the bilayer normal. This estimate is an upper limit, since part of the intensity probably came from mobile side chains. The estimate is consistent with the fact that Ile-13, the closest ^{15}N-labeled residue from Gly-18, was found to have a normal, perpendicular orientation from the bilayer normal based on the ^{15}N spectra.

In general, assignment of the ^{2}H spectrum of a globally exchange-labeled membrane peptide can only be made to a limited extent. Similar to the PISA wheel analysis for ^{15}N spectra (*16, 17*), one can rely on the known secondary structure of the peptide to calculate the coordinates of all N–D bonds, and convert these to ^{2}H spectral patterns for various helix orientations. These simulated spectra can then be compared with the experimental ^{2}H spectra to determine the actual helix tilt angle relative to the bilayer normal. However, information on the polarity of the helix and complete assignment cannot be obtained without site-specific deuteration of one of the N–D groups.

The practical limitations of the PRIDE technique for determining membrane peptide orientation are threefold. First, the extent of sensitivity enhancement is still below the estimated theoretical factor of about 25, mainly due to the t_1 noise at $\omega_2 = 0$ (*22*). This signal-associated noise results from the instabilities of the spectrometer and is much higher than the noise levels at other cross sections. Further sensitivity enhancement requires improved spectrometer stability. Second, the extraction of the orientational information relies on exchange labeling of the backbone amide hydrogen. Since the efficiency of ^{1}H/^{2}H exchange depends on various factors such as secondary structure, pH, and temperature, the equilibrium exchange level may vary from residue to residue and from peptide to peptide (*28*). This variation is not critical as long as one does not quantify the spectral intensities. One can maximize the ^{2}H exchange labeling by dissolving the peptide in a deuterated solvent prior to reconstituting it into the membrane (*28*). Third, the ^{2}H quadrupolar coupling constant is sensitive to hydrogen bonding (*25, 29*) and leads to uncertainties in the resulting angular constraints. Nevertheless, for helical peptides with similar hydrogen bonding patterns, this effect may be relatively constant. The gramicidin and ovispirin examples shown here provide benchmarks for the values of the quadrupolar splittings expected for transmembrane and surface-bound peptides, respectively.

The PRIDE approach combined with exchange labeling opens the possibility for measuring membrane peptide orientations in an economical fashion, without synthetic site-specific labeling. The large interaction strength of the ^{2}H quadrupolar coupling makes it sensitive to the orientation. PRIDE not only enhances the sensitivity of the ^{2}H spectra, but also suppresses the D_2O signal. This not only removes a severe dynamic range problem, but more importantly allows the detection of residues with the magic-angle orientation. Such detection of the magic-angle orientation is not possible by data processing methods or selective excitation methods (*18*), since they suppress all zero-frequency intensities in the spectrum indiscriminately. The PRIDE technique can also be applied to synthetically deuterated peptides (C–D bonds) to probe the segmental dynamics and side chain orientation of membrane peptides with increased sensitivity.

EXPERIMENTAL

Preparation of Oriented Samples

All deuterated solvents used here were purchased from Cambridge Isotope Laboratories (Andover, MA), and all lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Unlabeled gramicidin D, which is a mixture of 15-residue hydrophobic peptides, was purchased from Sigma (St. Louis, MO) and used without further purification. Twenty milligrams gramicidin D was dissolved in 1 ml CH_3CH_2OD and kept at 4°C in a closed container with small holes at the cap to recrystallize the peptide (*28*). After recrystallization, the peptide was dissolved in 1 ml of deuterated ethanol, CH_3CH_2OD (20 mg/ml). Then, 250 μl of the solution containing 5 mg of peptide were dried under N_2 gas and dissolved in 300 μl 2,2,2-trifluoroethanol-d$_3$(CF_3CD_2OD); 32.9 mg of 1,2-dilauroyi-*sn*-glycero-3-phosphocholine (DLPC) was dissolved in 100 μl deuterated chloroform ($CDCl_3$). The peptide and lipid solutions were mixed and the solution was deposited onto 24 glass plates of the dimension 16 × 4.5 × 0.08 mm (Electron Microscopy Sciences, Fort Washington, PA). The glass plates were dried in a N_2 atmosphere overnight and subsequently vacuum-dried using a water pump for 6 h. After elimination of the organic solvent, the glass plates were rehydrated in a closed chamber containing a saturated Na_2SO_4 solution prepared in D_2O (relative humidity: 95%) for at least two days. Then the plates were stacked and stored at 4°C in the same humidity until use.

Ovispirin (NH_2-KNLRRIIRKIIHIIKKYG-COOH) is a 18-residue antimicrobial peptide derived from sheep cathelicidins (*30*). Oriented ovispirin was prepared as recently described (*27*). Briefly, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were used to reconstitute the peptide; 2.5 mg of unlabeled ovispirin was dissolved in 600 μl protonated trifluoroethanol, and 22.5 mg of POPC/POPG mixture (3 : 1 molar ratio) was dissolved in 300 μl protonated chloroform. The two solutions were combined and deposited onto 24 thin glass plates of the dimension 16 × 4.5 × 0.15 mm. The glass plates were allowed to dry in air for 48 h, then they were rehydrated in a closed chamber containing a saturated Na_2SO_4 solution prepared in H_2O (relative humidity: 95%) for at least two days. Subsequently, the plates were stacked and kept at 4°C in a closed chamber containing a saturated D_2O solution of Na_2SO_4 until use. Ovispirin exchanged readily after membrane reconstitution in a protonated environment, in contrast to gramicidin. This is due to the surface orientation of ovispirin, which exposes it to the aqueous phase. In comparison, the transmembrane gramicidin has much reduced contact with D_2O once it binds to the lipid bilayer, thus exchange prior to membrane binding is important.

The POPC-d_{31} lipids were oriented using the same procedures as for the peptides. For the smallest amounts of lipid samples, only a single glass plate was used. These single-glass plate samples were sandwiched between Teflon blocks when positioned in the radio frequency coil.

Before the NMR experiments, the oriented membrane samples were wrapped in parafilm and sealed in polyethylene bags to prevent leakage of D_2O and back exchange of atmospheric H_2O.

NMR Spectroscopy

The NMR experiments were carried out on a Bruker DSX-400 spectrometer (Karlsruhe, Germany) equipped with a 9.4 T magnet. The Larmor frequencies are 400.49 MHz for ^{1}H and 61.48 MHz for ^{2}H. A home-built 16 × 4.5 × 4.5 mm rectangular silver coil was used for ovispirin, and a 16 × 4.5 × 3.5 mm coil was used for gramicidin and the pure lipid samples. The rectangular coils were placed in a Bruker double-resonance static probe originally equipped with a 5 mm diameter solenoid coil. Typical ^{2}H and ^{1}H 90° pulse lengths were 4.4 and 4.1 μs for ovispirin; pulse lengths were reduced to 3.3 and 3.8 μs for gramicidin due to the smaller size of the radio frequency coil.

Both the directly and indirectly detected ^{2}H spectra were acquired in the analog mode due to the large spectral widths involved. The directly detected ^{2}H spectra were acquired using the quadrupolar echo sequence (*31*). Typically, a recycle delay of 1 s and a spectral width of 500 kHz (for ovispirin) or 1 MHz (for gramicidin) were used.

For the 2D PRIDE experiments, the ^{2}H evolution time was incremented at 1 μs, giving a ^{2}H spectral width of 1 MHz. Only cosine-modulated data were collected, since the ^{2}H quadrupolar spectra of these uniaxially mobile peptides are symmetric with respect to the zero frequency. A real Fourier transform was used for the ^{2}H dimension of the 2D spectrum. One hundred twenty-eight t_1 increments were usually acquired in the 2D spectra. One hundred sixty and 112 scans were added per t_1 slice for ovispirin and gramicidin, respectively. The signal averaging was done in several 2D experiments to reduce the t_1 noise at the $\omega_2 = 0$ slice of the 2D spectra. Recycle delays from 2 to 2.8 s were used for the PRIDE experiments.

The excitation and reconversion of the ^{1}H–^{2}H heteronuclear multiple-quantum coherence (*32*) were achieved under ^{1}H homonuclear decoupling by the MREV-8 sequence (*33*) (Fig. 1). The 90° pulse length for the MREV-8 pulse train varied between 4 and 4.5 μs. The HMQC excitation and reconversion period each consisted of 8 MREV-8 cycles for gramicidin and 12 MREV-8 cycles for ovispirin.

The ^{1}H signal was detected in the windows of a pulsed spin–lock train to increase the sensitivity of the ^{1}H spectra under the static condition. The length of the spin–lock pulse was empirically adjusted to maximize the signal and was usually shorter than the ideal 90° pulse length (*34*). A predetection receiver dead time of 7–8 μs was used, followed by data sampling in 1.5 μs. One data point was acquired per window. For both peptides, 2048 windows of detection were used.

The PRIDE spectra were processed with 300 Hz of exponential broadening in the ^{1}H dimension. For the ^{2}H dimension, 1.5 kHz and 0.8 kHz of Gaussian broadening was applied for gramicidin and ovispirin, respectively. The 1D directly detected ^{2}H spectra were processed with Gaussian broadening of 0.8 kHz.

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