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SHORT COMMUNICATIONS

Orientation of the Antimicrobial Peptide, Cecropin A–Magainin 2 Hybrid, in a Lipid Bilayer Studied by ^{15}N Solid-State NMR

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Antimicrobial peptides have been found in a variety of organisms, including mammals, amphibians, and insects.[1,2] These natural antimicrobial peptides are known to play important roles in the host defense system and innate immunity.[1,2] The widespread increase of antibiotic-resistant bacterial and fungal strains has created considerable interest in using natural peptides as antibiotics.

Cecropin A, a cationic 37-amino acid antimicrobial peptide, was isolated from the hemolymph of the giant silk moth, *Hyalophora cecropi*.[3,4] Magainin 2, a 23-amino acid antimicrobaial peptide is found in the skin of the African clawed frog, *Xenopus laevis*.[5,6] Cecropin A and magainin 2 both display high lytic activity against Gram-negative bacteria, but have little hemolytic effect against human erythrocytes and other eukaryotic cells. Furthermore, a Cecropin–Magainin hybrid peptide, CAMA (KWKLFKKIGIGKFLHSAKKF), has more potent antibacterial activity and less hemolytic activity[7,8] than either the original cecropin A, or maganin 2 or the hemolytic peptide melittin.[7–9]

The structure of CAMA and its analogues bound to dodecylphosphocholine (DPC) micelles have been determined by solution NMR spectroscopy.[9] According to Oh *et al.*, CAMA has two amphipathic helices which are linked by a Gly–IIe–Gly sequence. The precise mode of interaction of CAMA with the lipid bilayers, however, has not been determined. To elucidate the molecular mechanisms of the antimicrobial activity of CAMA, it is important to determine the orientation of CAMA in lipid bilayers. Such structural information could also facilitate the design of new antimicrobial peptides combining desirable antimicrobial spectra, safety and potency. Solid-state NMR spectroscopy is a powerful tool for determining the orientation of membrane-active peptides and proteins in liquid-crystalline lipid bilayers.[10–12] It allows for the study of amorphous and partly mobile biological solids. To investigate the orientation of CAMA in lipid bilayers, we used ^{15}N solid-state NMR spectroscopy. Our approach, based on the orientation dependence of the NMR frequencies,[13–17] has been used to establish the orientation of various membrane-active peptides for example gramicidin A,[18] mastoparan[19] and melittin[20] in lipid bilayers.

EXPERIMENTAL

Peptide Synthesis

All the peptides listed in Table I were synthesized in a stepwise fashion on Fmoc–Gly–PEG–PS resin (PE Biosystems) using a Pioneer™ Peptide Synthesizer. The C-terminus of CAMA is usually amidated but for this study we removed the terminal amino group replacing it with a single glycine through an amide linkage. After synthesis, the peptides were cleaved from the resin by treatment with a mixture of TFA, phenol, triisopropylsilan and water (88:5:2:5 vol %) for 2 h at room temperature and the crude peptide was precipitated and washed repeatedly with cold diethylether before purification by HPLC on a Senshu-Pack reverse phase ODS column.

Table I. ^{15}N labeled CAMA peptides synthesized in this study

	Amino acid sequence
CAMA-L4	KWK[^{15}N]LFKKIGIGKFLHSAKKFG
CAMA-A17	KWKLFKKIGIGKFLHS[^{15}N]AKKFG
CAMA-F5A17	KWKL[^{15}N]FKKIGIGKFLHS[^{15}N]AKKFG

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Preparation of Oriented Samples

CAMA and lipid (DMPC/DMPG = 7:3) at a 1:10 molar ratio were dissolved in methanol/chloroform (2:1 v/v). The solution was spread onto scrupulously clean glass plates (5 × 9 × 0.1 mm) and dried under vacuum for 24 h. The dry plates were then stacked in a sample tube (6.5 mm external diameter and 20 mm length) containing a very small volume of water. (A water content in the CAMA/lipid 60% w/w.) The sample was capped with a teflon stopper held in place by a very small quantity of Araldite epoxy glue.[19] The sample tubes were incubated at 45 °C for 3–4 d.

Solid-State NMR Measurements

Solid-state ^{31}P and ^{15}N NMR spectra were obtained on a Chemmagnetics CMX 400 spectrometer operating at a resonance frequency of 397.80 MHz for ^{1}H, 161.04 MHz for ^{31}P, 40.31 MHz for ^{15}N. A static double-resonance probe equipped with a goniometer was used. Proton-decoupled ^{31}P and ^{15}N CP (cross polarization) NMR spectra were recorded for the oriented samples. For the ^{31}P NMR experiments, typical NMR parameters were 6.5 μs for the 90° pulse length, 4–5 ms contact time, 3 s cycle delay and 200–1,000 scans. The ^{31}P chemical shifts were referenced to H_3PO_4. For ^{15}N NMR experiments, typical NMR parameters were 6.0 μs for the 90° pulse length, 1ms contact time, 3 s cycle delay and 20,000–50,000 scans. The ^{15}N chemical shifts were referenced to $^{15}NH_4NO_3$ by setting the signal of solid $^{15}NH_4Cl$ to 18.0 ppm. Spectral simulations for the ^{15}N CSA (chemical shift anisotropy) analysis were carried out on a UNIX workstation (Silicon Graphics O2) using a FORTRAN 77 program as previously described.[17]

^{15}N CP NMR for Helix Orientation Determination

In this section, we introduce the principles of ^{15}N NMR spectroscopy for the determination of the helix orientation. Information on helix orientations in lipid bilayers can be extracted using the orientation dependency of the NMR frequencies. Specifically, the chemical shift frequency of an amide ^{15}N spin in a peptide depends on the angle β between the main principal axis (σ_{33} of the chemical shift tensor) and the external magnetic field (Figure 1). The σ_{33} axis of the ^{15}N chemical shift tensor is known to be ~17° from the N–H bond, based on model compound studies.[21,22] The N–H bond is in turn nearly parallel to the helix axis of α-helical peptides.[23] Thus, the σ_{33} axis of the ^{15}N chemical shift tensor is roughly collinear with the helical axis, while the tilt angle of the ^{15}N chemical shift tensor away from the axis perpendicular to the plane of the membrane (or from the magnetic field) is approximately equal to the tilt of the helix axis away from the axis perpendicular to the surface of the membrane. Due to such orientation, the ^{15}N signal resonates at a specific frequency determined by the helix axis orientation relative to the magnetic field. In a macroscopically oriented membrane when the bilayer is perpendicular to the magnetic field, the tilt of the helix away from the magnetic field is identical to the tilt angle of the ^{15}N chemical shift tensor away from the axis perpendicular to the plane of the membrane. As the axes of the helices in transmembrane proteins are approximately perpendicular to the plane of the membrane they can be easily distinguished from helical proteins oriented parallel to the membrane surface.

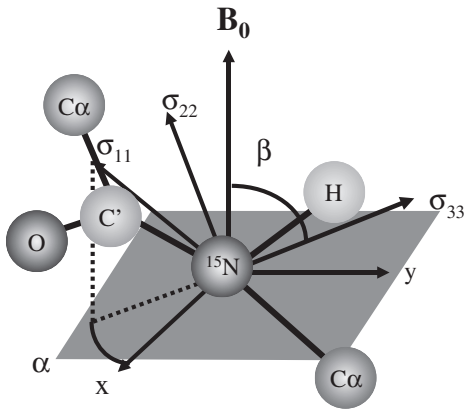

Figure 1. The orientation of ^{15}N chemical shift tensors, σ_{11}, σ_{22} and σ_{33} relative to the peptide N–C=O plane (membrane plane). The chemical shift of an amide ^{15}N spin in the peptide depends on the angle β between σ_{33} and the external magnetic field.

RESULTS

The determination of CAMA orientation requires the alignment of the lipid bilayers containing the peptide in a magnetic field. Therefore, at first, the degree of alignment was assessed by the ^{31}P spectra of the DMPC/DMPG lipids (Figure 2). When the lipid bilayers were unoriented, the ^{31}P spectrum showed a typical powder distribution (Figure 2a), characteristic of uniaxially oriented mobile molecules in randomly oriented bilayers. When the CAMA-bound lipid bilayers (Lipid:CAMA = 10:1) were oriented parallel to the glass plates and inserted into the magnet with the normal of the glass plates parallel to the magnetic field, the ^{31}P spectrum exhibited a single peak at the downfield edge (25.0 ppm) of the powder spectrum (Figure 2b below). This corresponds to a 0° angle between the motionally-averaged ^{31}P chemical shift tensor and the magnetic field. When the oriented sample was turned so that the alignment axis was perpendicular to the magnetic field, the ^{31}P peak shifted to the

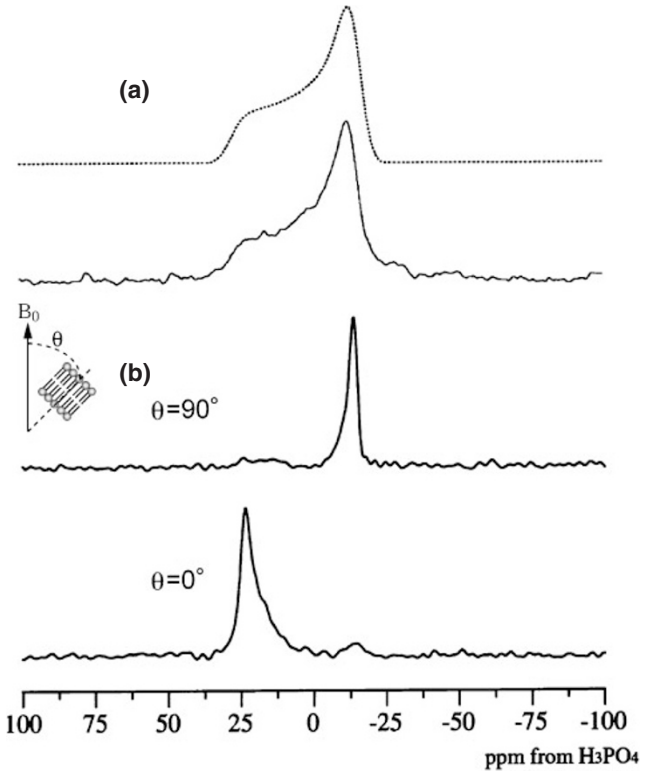

Figure 2. (a) The powder pattern ^{31}P spectrum of un-oriented DMPC/DMPG lipids (solid line) and simulated spectral pattern for determination of the chemical shift tensors (broken line). (b) The ^{31}P solid state NMR spectra of oriented CAMA-bound lipid bilayers (Lipid:CAMA = 10:1) when the acyl chains of the lipid molecules were set parallel ($\theta = 0°$) and perpendicular ($\theta = 90°$) to the external magnetic field.

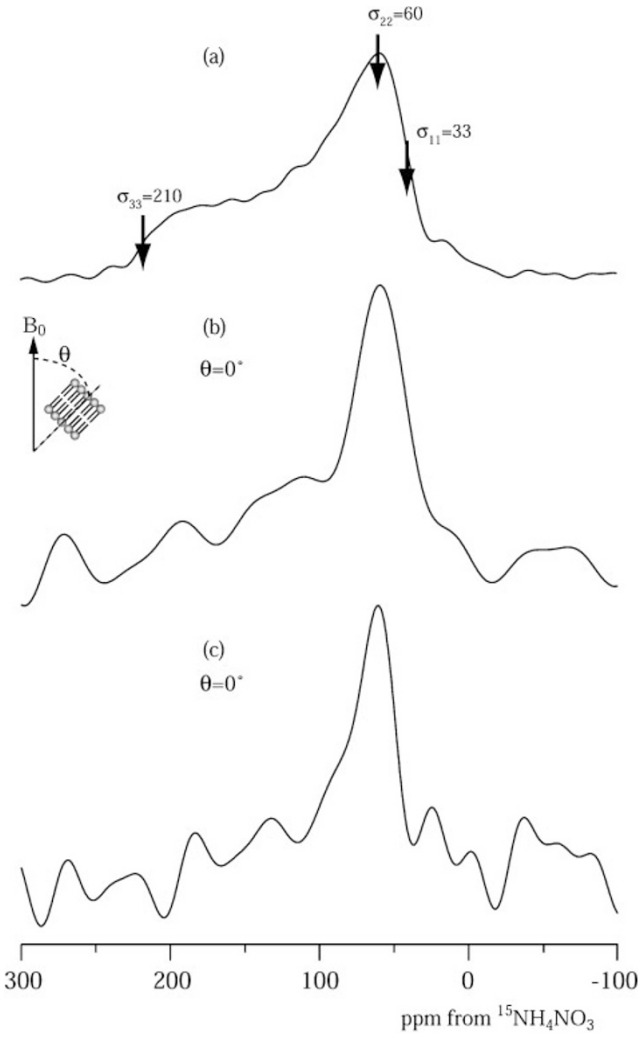

Figure 3. (a) The ^{15}N solid-state NMR spectrum of un-oriented CAMA peptide where the ^{15}N chemical shift tensors were determined to be $\sigma_{11} = 33$ ppm, $\sigma_{22} = 60$ ppm, and $\sigma_{33} = 210$ ppm. The ^{15}N solid-state NMR spectra of (b) ^{15}N-Leu-labeled CAMA-L4 and (c) ^{15}N-Ala-labeled CAMA-A17 molecules oriented in DMPC/DMPG lipid bilayers. ^{15}N single peaks at 60 ppm were observed when the bilayer normal was parallel to the external magnetic field.

upfield edge (−15.0 ppm) of the powder spectrum (Figure 2b upper). Thus, the CAMA molecules embedded in the bilayers were well-oriented.

^{15}N-labeled CAMAs were synthesized to determine the orientation of N and C-terminal amphipathic helices in lipid bilayers using samples aligned on glass plates. Knowledge of the magnitude and orientation of the chemical shift tensor in the molecular frame is necessary to interpret the ^{15}N chemical shift spectra of the aligned samples. These parameters were obtained from a dry powder sample of CAMA-F5A17. From the ^{15}N chemical shift powder pattern (Figure 3a), the principal values of the ^{15}N chemical shift tensor were determined as $\sigma_{11} = 33$ ppm, $\sigma_{22} = 60$ ppm, and $\sigma_{33} = 210$ ppm.

Solid-state NMR spectra obtained for the ^{15}N-labeled CAMA peptides in DMPC/DMPG bilayers are shown in Figure 3. Both CAMA-L4 and CAMA-A17 molecules in DMPC/DMPG lipid bilayers displayed a ^{15}N single peak at 60 ppm when the plane of the bilayer was perpendicular to the magnetic field (Figures 3b, c). This chemical shift value indicates that the NH bond is parallel to the membrane plane. Thus, both CAMA-L4 and CAMA-A17 ^{15}N single peaks are observed in the upfield (σ_{11}, σ_{22}) region in the amide ^{15}N chemical shift powder pattern, indicating that both helices are oriented parallel to the membrane plane because Leu 4 and Ala 17 were located at the N- and C-terminal helices, respectively.

The ^{15}N peak of CAMA-F5A17 also resonated at 60 ppm when the bilayer was perpendicular to the magnetic field (Figure 4a). When the bilayer normal is perpendicular to the magnetic field, however, CAMA-F5A17 displays chemical shifts of 54 and 162 ppm. This orientation dependency of the spectral change can be simulated using such parameters as ^{15}N chemical shift tensor elements ($\sigma_{11} = 33$, $\sigma_{22} = 60$, $\sigma_{33} = 210$), Euler angles α and β, and distributions of the helix order parameter p.[15] Excellent fit

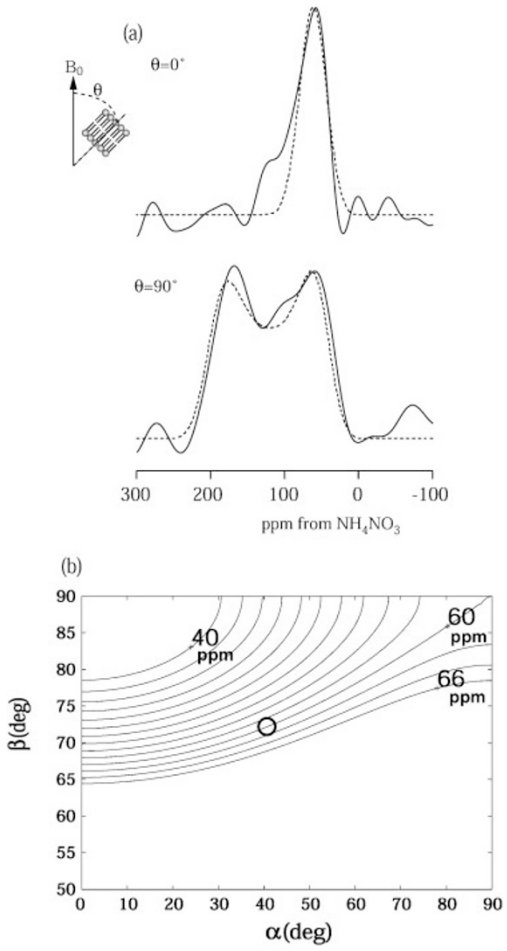

Figure 4. (a) The ^{15}N solid-state NMR spectra of ^{15}N-Phe and ^{15}N-Ala double labeled CAMA-F5A17 molecule oriented in DMPC/DMPG lipid bilayers when the acyl chains of the lipid molecules were set parallel ($\theta = 0°$) and perpendicular ($\theta = 90°$) to the external magnetic field. (b) The spectra were simulated with parameters such as ^{15}N chemical shift tensors ($\sigma_{11} = 33$ ppm, $\sigma_{22} = 60$ ppm, $\sigma_{33} = 210$ ppm), Euler angles α and β, and distribution of helix order parameter p.[15] The numbers inserted mean the calculated ^{15}N chemical shift values in ppm when the bilayer normal was parallel to the external magnetic field. Excellent fit to the experimental spectra (broken lines in (a)) was obtained when $\alpha = 41°$, $\beta = 71°$ and $p = 5°$ (open circle).

to the experimental spectra was obtained when $\alpha = 41°$, $\beta = 71°$ and $p = 5°$ (Figure 4b). From these parameters, we were able to determine the orientation of the average CAMA helix axes relative to the membrane plane.

DISCUSSION

The orientation of CAMA peptide in lipid bilayers was investigated by ^{15}N NMR spectroscopy, utilizing the fact that the orientation of a helical peptide in lipid bilayers can be deduced from ^{15}N chemical shift anisotropy. The amino acid sequence of CAMA is KWKLFKKIGIGKFLHSAKKF(NH_2), where the underlined Gly–Ile–Gly hinge region links the N- and C-terminal helices. The secondary structure of CAMA is known to be α-helical from solution NMR spectroscopy.[9] We prepared two CAMA samples, specifically ^{15}N-labeled at either Leu4 at the N-terminal helix or Ala17 in the C-terminal helix. From the orientation dependent ^{15}N NMR experiments, we determined the orientation parameters, $\alpha = 41°$ and $\beta = 71°$ by spectral simulation. Using these parameters, the orientations of the CAMA helices orientation in lipid bilayers could be estimated. The helices of CAMA were found to be tilted by about 70° from the bilayer normal. That is, the helices are tilted by about 20° from the membrane plane. The arrangement of CAMA in DMPC/DMPG lipid bilayers is shown schematically in Figure 5. In this arrangement, the hydrophobic residues of CAMA are thought to make contact with the lipid acyl chain region, while the hydrophilic residues face toward the lipid–water interface.

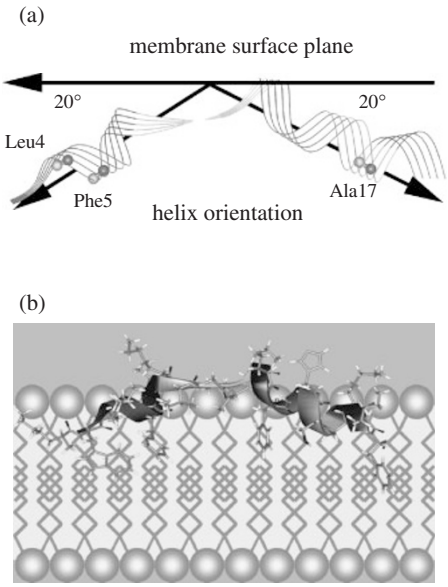

Figure 5. The arrangement of a CAMA molecule in DMPC/DMPG lipid bilayers determined in this work. In this arrangement, the hydrophobic residues of CAMA make contacts with the lipid acyl chain region, while the hydrophilic residues face toward the water–lipid interface.

The mode of orientation of peptides parallel to the membrane surface is characteristic of the "carpet" model mechanism.[24] Several membrane active peptides are known to take the "carpet" model orientation, for example, cecropin A,[25] ovispirin,[26] dermaseptin S,[27] and human antimicrobial peptide LL-37.[28] According to this model, peptides bind onto the surface of the target membrane and cover the surface. Initial interaction is by electrostatic binding of the peptide to the negatively charged membrane surface. This may explain their effectiveness against the difficult-to-combat gram negative organisms with

their acidic coats. Later when higher peptide concentrations have accumulated they cause membrane permeation leading to membrane disintegration. CAMA has seven lysine residues, and is therefore likely to bind electrostatically to the negatively charged membrane surface. Moreover, CAMA has hydrophobic amino acids, Trp2, Phe5, Phe14 and Phe20, the bulky hydrophobic side chains of which interact with the lipid acyl chain region. In addition, CAMA has a flexible hinge region in its secondary structure and this seems to cause effective membrane perturbation for reasons that are not entirely understood.

Thus ^{15}N solid-state NMR can add to our understanding of the interaction of antimicrobial peptides with bacterial membranes and help to explain the mechanism of action of these potentially important peptides.

CONCLUSIONS

The orientation of CAMA peptide in lipid bilayers was determined from the ^{15}N CSA obtained from ^{15}N solid-state NMR experiments. The results presented here indicate that both the C- and N-terminal helices of CAMA have a tilt angle of 20° from the lipid bilayer plane. This orientation suggests that CAMA adopts a "carpet" model when interacting with bacterial membranes.

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