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ARTICLE

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Solid-state NMR study of antimicrobial peptides from Australian frogs in phospholipid membranes

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Abstract Antimicrobial peptides, isolated from the dorsal glands of Australian tree frogs, possess a wide spectrum of biological activity and some are specific to certain pathogens. These peptides have the capability of disrupting bacterial membranes and lysing lipid bilayers. This study focused on the following amphibian peptides: (1) aurein 1.2, a 13-residue peptide; (2) citropin 1.1, with 16 residues; and (3) maculatin 1.1, with 21 residues. The antibiotic activity and structure of these peptides have been studied and compared and possible mechanisms by which the peptides lyse bacterial membrane cells have been proposed. The peptides adopt amphipathic α-helical structures in the presence of lipid micelles and vesicles. Specifically ^{15}N-labelled peptides were studied using solid-state NMR to determine their structure and orientation in model lipid bilayers. The effect of these peptides on phospholipid membranes was determined by ^{2}H and ^{31}P solid-state NMR techniques in order to understand the mechanisms by which they exert their biological effects that lead to the disruption of the bacterial cell membrane. Aurein 1.2 and citropin 1.1 are too short to span the membrane bilayer while the longer maculatin 1.1, which may be flexible due to the central proline, would be able to span the bilayer as a transmembrane α-helix. All three peptides had a peripheral interaction with phosphatidylcholine bilayers and appear to be located in the aqueous region of the membrane bilayer. It is proposed that these antimicrobial peptides have a ''detergent''-like mechanism of membrane lysis.

Keywords Antibiotics · Bilayers · Membranes · Peptides · Solid-state NMR

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Introduction

Antimicrobial peptides are found in both invertebrates (Boman 1991) and vertebrates, including humans (Agerbeth et al. 1995; Gudmundsson et al. 1996), and in insects, amphibians and plants (Jacob and Zasloff 1994). These antimicrobial peptides are an important component of immune defence systems. The necessity of developing a new generation of antibiotics to overcome resistance by certain pathogens to conventional antibiotics is of increasing importance. However, most antimicrobial peptides are toxic to a variety of microorganisms as well as mammalian cells, e.g. melittin isolated from bee venom (Habermann and Jentsch 1967) and cecropin-like human LL-37 (Johansson et al. 1998; Oren et al. 1999). Mechanisms by which these peptides disrupt bacterial cells have been proposed, including the ''barrel stave'' and ''carpet'' models (Ehrenstein and Lecar 1977; Shai 1999; Shai and Oren 2001). Previous studies indicate that most antimicrobial peptides interact directly with the membrane of the microorganism and can permeate the bilayer, leading to collapse of the membranes. These peptides are positively charged due to the presence of amino acids such as lysine and arginine, and contain a high percentage of hydrophobic amino acids (Oren et al. 2002). It has been proposed that these positively charged peptides preferentially bind to the negatively charged outer surface of bacteria that contain lipopolysaccharides or Gram-positive, acidic polysaccharides (Brock 1974).

Antimicrobial peptides isolated from the dorsal glands of Australian amphibians possess a wide spectrum of biological activity and are specific to certain pathogens (Stone et al. 1992a, 1992b; Steinborner et al.

1998; Wegener et al. 1999; Chia et al. 2000a; Rozek et al. 2000). These peptides from the tree frogs *Litoria aurea*, *L. citropa* and *L. genimaculata* (Wegener et al. 1999; Chia et al. 2000a; Rozek et al. 2000) vary from 10 to 25 amino acid residues (Table 1). Solution NMR and circular dichroism spectra indicate a similar amphipathic helical conformation for these peptides, which have the capability of disrupting bacterial membranes and lysing lipid bilayers.

To understand the mechanism of these membrane-active peptides, aurein, citropin and maculatin have been studied using solid-state NMR spectroscopy to determine the location of labelled peptides within phospholipid bilayers. We have studied the interaction of these peptides with deuterated dimristyolphosphatidylcholine using NMR to determine the mechanism of membrane disruption and lysis. The structure of the amphibian peptides aurein 1.2, citropin 1.1 and maculatin 1.1 in phospholipids were compared using solid-state NMR techniques, specifically ^{15}N-labelled peptides in aligned phospholipid bilayers oriented at 0° and compared to an earlier study (Marcotte et al. 2003) where the bilayers were studied at 90° to the magnetic field. The results demonstrate the importance of studying aligned systems at both angles, particularly when the chemical shift anisotropy is small.

Aurein 1.2 consists of 13 residues with the sequence GLFDIIKKIAESF-NH_2, citropin 1.1 has 16 amino acid residues with the sequence GLFDVIKKVASVIGGL-NH_2 and maculatin 1.1 with 21 residues has the sequence GLFGVLAKVAAHVVPAIAEHF-NH_2. At the C-terminus, these peptides have the same three amino acids (glycine, leucine and phenylalanine), while aurein and citropin also share the fourth amino acid, aspartate. Both aurein and maculatin have phenylalanine at the N-terminus. Comparison of the sequence of aurein and citropin shows similarity in charged residues, which are important for biological interaction. The aurein peptide contains two negatively charges amino acids (aspartic and glutamic acid) at the N- and C-termini, while citropin contains only one negative charged residue at the N-terminus, i.e. aspartic acid. A notable observation is the presence of two positive residues (lysine) adjacent to each other at positions 7 and 8 in both aurein and citropin, while maculatin has a lysine at position 8. Location of positively charged amino acids in the centre with negative charges at the end of the peptide sequence may play an important role for electrostatic interactions between amphipathic peptides and membrane bilayers, for example magainin from frogs and melittin from bees are lytic peptides that interact electrostatically with membranes (Bechinger et al. 1991; Smith et al. 1994). Maculatin has a proline at position 15 with a pronounced kink and flexible hinge region that may enable amphipathic distribution of amino acids and a more precise interaction of the peptide with the membrane surface (Wong et al. 1997). Aurein and citropin are shorter peptides with similar biological activity to maculatin but without a proline residue. However, the amino acid distribution of these shorter peptides (Wegener et al. 1999) may not require central flexibility to enhance their interaction with phospholipid membranes. Overall, aurein, citropin and maculatin are basic peptides that may interact electrostatically with anionic phospholipids (Chia et al. 2000b) and less so with zwitterionic or neutral phospholipids.

Materials and methods

Citropin 1.1 with [^{15}N]Ala10 or [^{15}N]Gly14, aurein 1.2 with [^{15}N]Ala10 and maculatin 1.1 with [^{15}N]Ala10 were synthesized by Mimotopes (Melbourne, Australia). The purity of the labelled peptides was >90%. Deuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_{54}) was obtained from Avanti Polar Lipids (Alabaster, USA).

Sample preparation

The unoriented samples were prepared by dissolving the peptide (~6 mg) and phospholipid (DMPC-d_{54}) (1:10 molar ratio) in 600 μL of methanol/chloroform (1:1 v/v). The solution was dried under a stream of nitrogen gas and then vacuum pumped overnight. The dry powder was studied in both the dehydrated and hydrated form. The powder sample in a 5-mm NMR rotor was hydrated by addition of water (~60 μL). Oriented samples were prepared as described previously (Cornell et al. 1988; Smith et al. 1994) by dissolving the phospholipid and peptide in ~1 mL methanol/chloroform (1:1 v/v). The solution was applied to ~25 thin glass plates, allowed to evaporate and then vacuum pumped overnight. The samples were then hydrated with water by applying one drop of water (~2 μL) on top of each glass slide and the slides stacked. Any excess sample mixture at the edges of the glass plates was carefully removed. The plates were inserted into a 7-mm diameter NMR tube containing 10 μL of water, and then heat sealed. The sample tube was mounted horizontally into a static NMR probe with the normal to the plates oriented parallel to the applied magnetic field (0° orientation).

NMR experiments

All experiments were carried out on a Varian (Palo Alto, USA) Unity Inova 300 spectrometer operating at resonance frequencies of 300.01 MHz for ^{1}H, 121.46 MHz for ^{31}P, 75.45 MHz for ^{13}C, 46.06 MHz for ^{2}H and 30.41 MHz for ^{15}N. A magic angle spinning (MAS) probe with 5 mm sample diameter was used for unoriented

Table 1 Amino acid sequence and biological activity comparison of amphibian peptides

Peptide	Amino acid sequence	*Bacillus cereus* MIC (μg mL^{-1})[a]
Aurein 1.2	GLFDIIKKIAESF-NH_2	5
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	50
Maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF-NH_2	25

[a]Wegener et al. (1999); Rozek et al. (2000); Chia et al. (2000a)

samples, and a static probe with 7 mm diameter was used for oriented samples. For ^{31}P, a 4 μs 90° pulse, 2 s recycle time and 100 kHz sweep width was used. Spectra were acquired using both a single pulse and a spin-echo sequence with 40 μs echo delay, and proton decoupling. ^{13}C and ^{15}N spectra were obtained using cross polarization (CP) (Pines et al. 1973) with 90° pulse times of 4 μs and 8 μs, recycle times of 2 s and 1.5 s, a sweep width of 50 kHz and 30 kHz, respectively, and a contact time of 1.5 ms. For ^{2}H spectra, a solid echo (Mansfield 1965) was used with a 4.3 μs 90° pulse, 0.5 s recycle time, a 100 kHz sweep width and a 30 μs echo time. Line broadening of 100 Hz was applied. The ^{15}N, ^{13}C and ^{31}P chemical shifts were referenced externally to a saturated $^{15}NH_4NO_3$ solution, TMS or 85% H_3PO_4 (0 ppm), respectively. All spectra were acquired at 28–30 °C to ensure that the lipids were in the liquid-crystalline phase.

Results and discussion

Unoriented samples

^{31}P NMR spectra of hydrated powders of peptides reconstituted into DMPC-d_{54} (1:10 molar ratio) are shown in Fig. 1. The ^{31}P chemical shift anisotropy (CSA) at 30 °C was approx. −35 ppm, with maximum intensity at approximately −12 ppm, indicating a fluid membrane bilayer. The CSA was less than for DMPC-d_{54} alone (approx. −45 ppm). ^{31}P MAS NMR spectra resulted in a narrow single peak with a chemical shift position at −1.3 ppm, typical for DMPC.

^{2}H NMR spectroscopy was used to check the effect of these peptides on deuterated DMPC to determine quadrupolar splittings and, hence, order parameter changes. The increase or decrease in the quadrupolar splitting or order parameter of phospholipid bilayers after addition of peptide correlates with changes in thickness of the membrane bilayer (Bechinger et al. 1991; de Planque et al. 1998; Nagle and Tristram-Nagle 2000). ^{2}H NMR spectra of hydrated powder samples of peptides incorporated into deuterated phospholipid bilayers obtained at 30 °C are shown in Fig. 2.

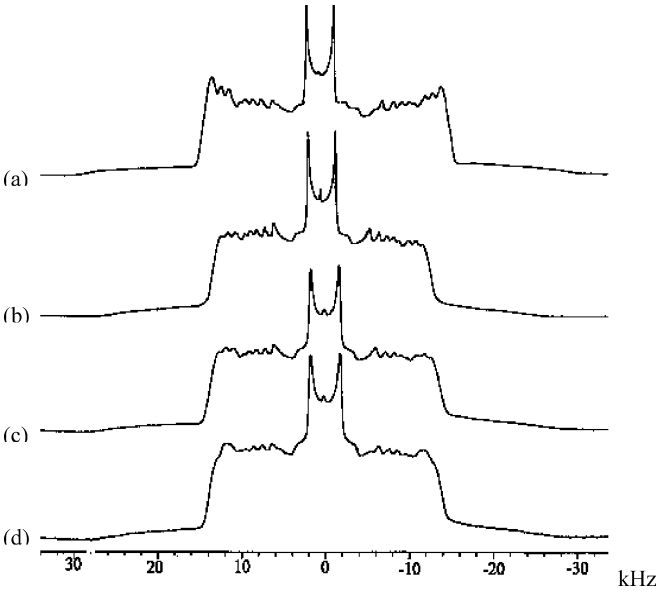

Fig. 2 ^{2}H NMR spectra of hydrated dispersions of peptides in deuterated DMPC: (*a*) DMPC-d_{54}, (*b*) aurein 1.2, (*c*) citropin 1.1 and (*d*) maculatin 1.1. Typically, 28,000 scans were acquired at 30 °C

In these spectra, the methyl (CD_3) signals are well resolved, with quadrupolar splittings of ∼3 kHz, while the methylene (CD_2) signals were not completely resolved. The measured maximum quadrupolar splitting ($\Delta\nu_Q$) at the 90° edge for maculatin decreased slightly to ∼28 kHz, with a more significant decrease in the case of citropin and aurein (∼27 kHz and ∼26 kHz splittings, respectively) compared to ∼29 kHz for DMPC multilayers. The bond order parameter (S_{CD}) can be calculated according to Seelig (1977). The order parameters are proportional to the quadrupolar splittings, which are larger for sites closer to the glycerol backbone and decrease down the acyl chain toward the centre of the

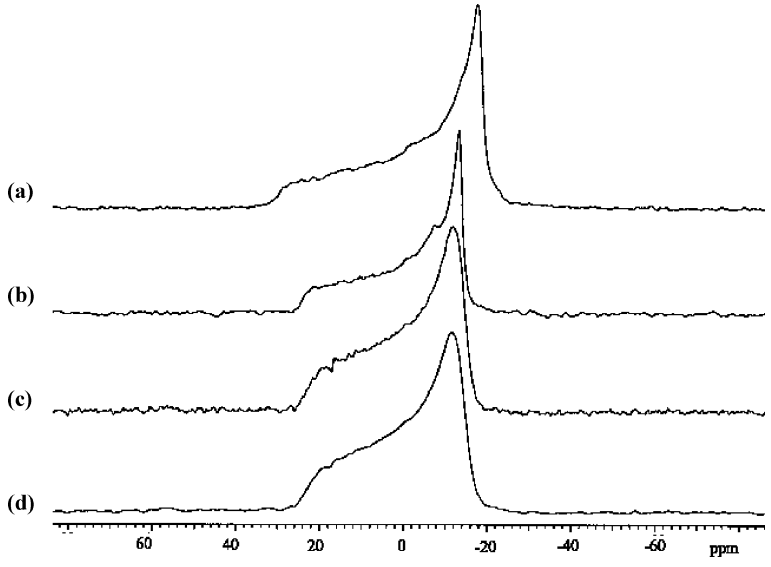

Fig. 1 ^{31}P NMR spectra of hydrated dispersions of (*a*) DMPC-d_{54} alone and with (*b*) aurein 1.2, (*c*) citropin 1.1 and (*d*) maculatin 1.1. The typical number of scans was 2500 acquired at 30 °C

bilayer (Seelig and Seelig 1974). There was a small decrease (5–10%) in the lipid bilayer order after incorporation of the amphibian peptides.

CP MAS ^{15}N NMR spectra obtained at 28 °C of dry powder samples with aurein, citropin or maculatin incorporated into DMPC-d_{54} resulted in a chemical shift with an isotropic value of ~100 ppm for the alanine residues (Fig. 3). An isotropic value of ~100 ppm is indicative of an α-helical peptide (Shoji et al. 1987, 1989, 1990). In one case, ^{15}N-labelled citropin incorporated into lipid and freeze dried showed two resonances under MAS conditions, with chemical shifts of 99 ppm and 107 ppm, indicative of α-helix and β-sheet conformations, respectively, with the α-helical form having greater intensity. When the sample was hydrated, incubated at 45 °C for 5 h and freeze dried again, the smaller β-sheet component disappeared. In every other case, the ^{15}N (or ^{13}C) MAS chemical shift indicated an α-helical conformation. In the presence of phospholipid bilayers, even in the dry state these peptides favour an α-helical structure, as previously shown using circular dichroism (Marcottte et al. 2003).

Oriented samples

Solid-state ^{15}N and ^{13}C NMR supported by ^{2}H and ^{31}P NMR spectroscopy is used to study the orientation of membrane-active peptides in mechanically aligned phospholipid bilayers. Aligned membrane bilayers incorporating ^{15}N specifically labelled peptides are used to determine the orientation of α-helical peptides (Bechinger et al. 1991). ^{15}N chemical shifts of ~210 ppm for oriented peptides are indicative of a transmembrane insertion, whereas < 100 ppm is characteristic of peptides oriented on the membrane surface (Bechinger et al. 1991; Harzer and Bechinger 2000). Accordingly, the orientation of several membrane-active peptides has been determined by ^{13}C and ^{15}N NMR (Cornell et al. 1988; Smith et al. 1994; Cross 1997; Marassi et al. 1997). The orientation of specifically labelled peptides, namely aurein, citropin and maculatin labelled at the 10th, 11th or 14th residue, was investigated by ^{15}N solid-state NMR techniques. Cross polarization was used to enhance the rare spin signal and allow faster acquisition due to the shorter proton relaxation times (Pines et al. 1973). ^{31}P and ^{2}H solid-state NMR spectra of aligned membranes were recorded at 30 °C to determine the effect on the phospholipid head-group and acyl chain order, respectively.

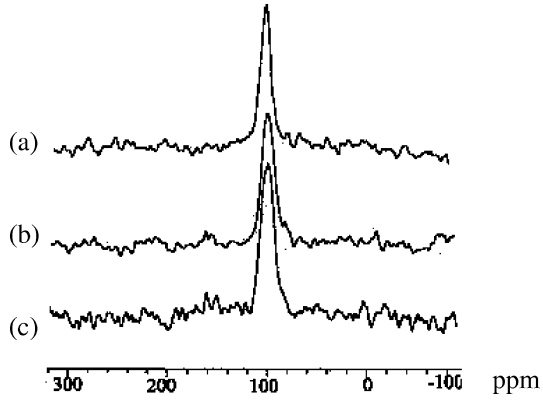

Fig. 3 ^{15}N NMR spectra of (*a*) aurein 1.2, (*b*) citropin 1.1 and (*c*) maculatin 1.1 in DMPC-d_{54}. Approximately 50,000 scans were recorded at 28 °C

^{2}H NMR spectra of aligned samples of deuterated DMPC were recorded at both 0°and 90° orientations with and without peptide. The quadrupolar splitting at 0° is double the 90° value, and in the case of DMPC-d_{54} aligned bilayers with and without maculatin, came to ~56 kHz and ~57 kHz, respectively, in accordance with the powder spectra. The use of aligned spectra oriented at 0° to the magnetic field is equivalent to dePaking the powder spectra (Separovic and Gawrisch 1996). ^{2}H NMR spectra of aligned samples with citropin and aurein reconstituted in phospholipid bilayers, like the hydrated dispersions, showed a decrease in quadrupolar splitting or order parameter (Fig. 4) with values of ~52 kHz and ~49 kHz, respectively. This may indicate that these two amphipathic α-helical peptides may adopt an in-plane mode of action (Hoffman et al. 1983; Gawrisch et al. 1995; Bechinger et al. 1998; Bonev et al. 2000). These peptides are too short to span the membrane and may possibly lie on the membrane surface. ^{2}H NMR spectra of maculatin–lipid bilayers had a lesser effect on the overall quadrupolar splitting, which may indicate a transmembrane orientation. Since maculatin 1.1 is a longer peptide with 21 residues, the peptide may cross the membrane and possibly interact with the lipid membrane according to the proposed barrel-stave mechanism. The aligned bilayers may aid insertion of the longer peptide into the lipid membranes, as is the case with melittin (Smith et al. 1994). Note that in both Figs. 2 and 4 there is a decrease in resolution (or T_2) of the quadrupolar splittings for the bilayers with amphibian peptides, and this decrease in T_2 is greater in the case of maculatin and citropin, suggesting greater interaction with the phospholipid for the longer peptides. This decrease in T_2 is also evident in the ^{31}P spectra of Fig. 1, where the change in the CSA is convoluted with a change in T_2, indicating an overall slowing down of the phospholipids in the presence of the peptides. The decreased CSA, however, indicates a disordering of the phospholipid head-groups by the peptides.

^{31}P NMR spectra at 0° and 90° orientations of the aligned bilayers to the magnetic field were acquired to gain further information on the phospholipid headgroups in the presence of the amphipathic peptides. ^{31}P chemical shift of value ~23 ppm at 0° orientation indicated that the majority of the phospholipids were aligned with their bilayer parallel to the magnetic field, although significant powder signals at an upfield resonance frequency (~−12 ppm) were observed (Fig. 5). The peptides appeared to disorder the phospholipid

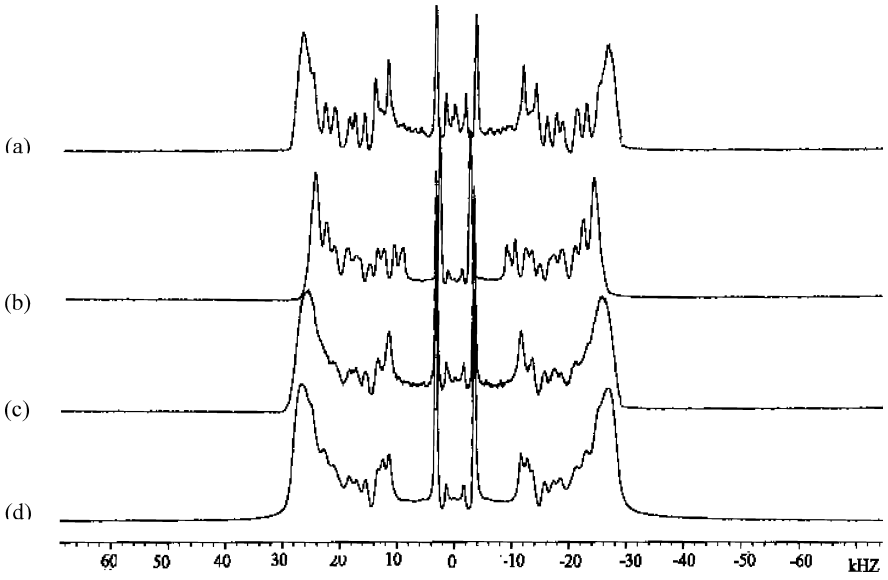

Fig. 4 ^{2}H NMR spectra of (*a*) DMPC-d_{54} oriented with the bilayer normal parallel to the magnetic field, and with (*b*) aurein 1.2, (*c*) citropin 1.1 and (*d*) maculatin 1.1. Typically, 26,000 scans were acquired at 30 °C

head-group since, as seen in Fig. 1 for hydrated dispersions also, the CSA was reduced compared to pure DMPC-d_{54} in aligned bilayers, especially in the case of aurein. The difference in CSA between the hydrated dispersions (Fig. 1) and the aligned samples (Fig. 5) most likely results from the different amounts of water between the powder and oriented systems. Aligned bilayers support about 25 waters per DMPC molecule (Cornell et al. 1988), while the hydrated dispersions would have excess water present. The aligned systems may trap the water-soluble peptide molecules between the multilayers and increase the interaction with the phospholipid headgroups. Aurein samples showed a greater increase in the isotropic ^{31}P NMR resonance (δ_{iso}) with time by comparison with citropin and maculatin, which is an indication of a loss in membrane stability, and may reflect the greater potency of aurein (Table 1). A previous ^{31}P NMR study has shown such an effect by amphibian peptides on the ^{31}P spectra on bacteria (Chia et al. 2000b). There was a small suggestion of an isotropic peak in the hydrated dispersions with aurein (Fig. 1), and since the isotropic peak intensity increased with time, disruption of the membrane bilayer occurred over the long acquisition period required for acquiring ^{15}N spectra of the aligned samples. Freeze-thawing the samples reduced the size of the isotropic peak, which suggests that lipid degradation was not the cause of the isotropic signal. Similar effects were seen with maculatin (Marcotte et al. 2003) and citropin following long acquisition times and sample storage.

^{15}N solid-state NMR spectroscopy was used in order to determine the orientation of these membrane-active peptides in aligned DMPC-d_{54} bilayers. Peptides were specifically labelled along the backbone. [^{15}N]Ala-10-labelled aurein 1.2, citropin 1.1 and maculatin 1.1 were used as well as [^{15}N]Gly-14 citropin 1.1. Owing to molecular motion, it was difficult to cross polarize (Hong et al. 2002) from protons to ^{15}N both in the hydrated powder dispersions and aligned lipid multilayers. NMR results obtained from oriented samples in other

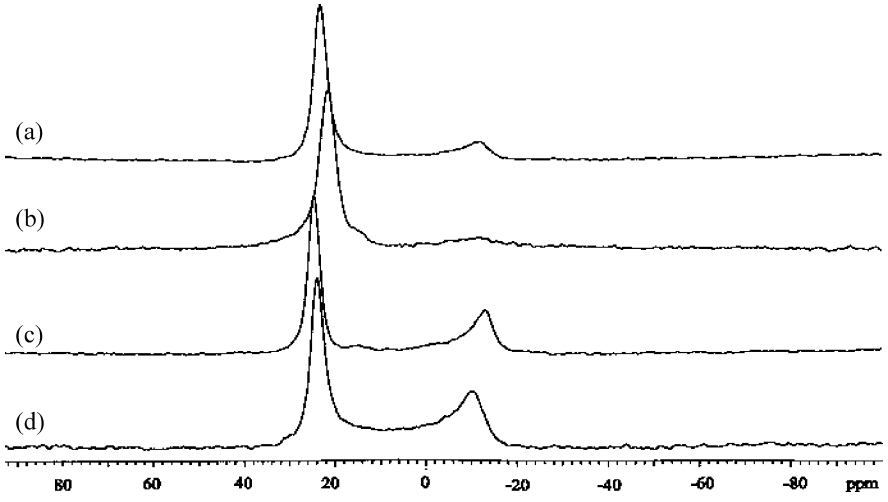

Fig. 5 ^{31}P NMR spectra of peptides aligned in DMPC-d_{54}: (*a*) DMPC-d_{54} alone, and with (*b*) aurein, (*c*) citropin and (*d*) maculatin, oriented at 0° to the magnetic field. Typically, 3500 scans were acquired at 30 °C

studies have shown that incorporation of similar peptides (e.g. cecropin, magainin, melittin and protegrin-1) into lipid bilayers could be via the barrel-stave mechanism (He et al. 1996; Duclohier and Wroblewski 2001) or the peptides may interact with the external surface of the membrane by electrostatic interaction between positively charged peptides and anionic lipid head-groups (Bechinger et al. 1991, 1998; Marassi et al. 1999). Our study in neutral lipids would better reflect the interaction of these peptides on red blood cells rather than bacteria.

Our MAS results for the peptides gave ^{15}N chemical shifts of ~100 ppm for the alanine residues (and ~90 ppm for the glycine residue) in dry lipid powders (Fig. 3), indicating an α-helical conformation. In the case of aurein 1.2, a signal was obtained at ~100 ppm in hydrated powders and in aligned bilayers at 0° to the magnetic field (Fig. 6). This indicates that aurein interacts weakly with the lipid bilayers, and may be mobile in the aqueous phase trapped between the bilayers. The peptide may be partially incorporated into the bilayer, but since the chemical shift is close to the isotropic value both in aligned and unoriented hydrated dispersions, the peptides appear to be mobile and/or reorienting close to the magic angle (50–55°) relative to the bilayer normal. Lowering the temperature to 10 °C had little effect on the ^{15}N spectra of aurein as a hydrated dispersion or in an aligned sample. Since deuterated DMPC has a gel–fluid phase transition temperature at about 20 °C, the lack of effect on the ^{15}N spectrum of aurein indicates that the peptide is mobile in the aqueous phase. The difficulty with CP also suggests that the peptides are mobile, and since similar chemical shifts were obtained for aligned citropin and aurein bilayers at 90° to the field (Marcotte et al. 2003), the peptides appear to be in the aqueous phase between the bilayers. In our earlier study (Marcotte et al. 2003), aligned spectra were only obtained at 90° to the magnetic field. The present results obtained using samples aligned at 0° to the field (Fig. 6b) had a similar chemical shift (~ 100 ppm). This chemical shift is the same as obtained for aurein in both dry and hydrated DMPC powders (Fig. 3a and Fig. 6a) under MAS and static conditions. Since a chemical shift very similar to the isotropic value was obtained, the ^{15}N data indicate that the peptide aurein is mobile in the aqueous phase in hydrated DMPC multilayers. The results illustrate the importance of obtaining the chemical shift at both 0° and 90° orientations for aligned samples, particularly in cases where the CSA is small. A combination of mobility and alignment at the magic angle (Hong et al. 2002) would explain the difficulty in obtaining CP spectra from the aligned maculatin samples. Results gained using [^{13}C = O]Ala-11 maculatin 1.1 indicated that the peptide was mobile in hydrated DMPC dispersions. Although ^{13}C NMR is more sensitive than ^{15}N, owing to the higher natural abundance, a large background ^{13}C signal from peptide and lipid carbonyls was obtained. Similar to the ^{15}N results, under MAS conditions in dry and hydrated lipid powders an isotropic chemical shift of 176 ppm was obtained, indicating an α-helical peptide. Despite the large ^{13}C background, a significantly reduced ^{13}C CSA obtained under static conditions indicated that maculatin was mobile in hydrated phospholipid dispersions (data not shown). The difficulty in obtaining a ^{15}N spectrum of maculatin in hydrated bilayers despite several attempts is most likely due to high molecular mobility, leading to poor CP efficiency. In the case of aurein, we were fortunate to find the right CP conditions to match the ^{15}H and ^{1}H transmitters over a very narrow power range.

Although only the data for ^{15}N-labelled aurein indicate that this peptide is mobile in the aqueous phase of

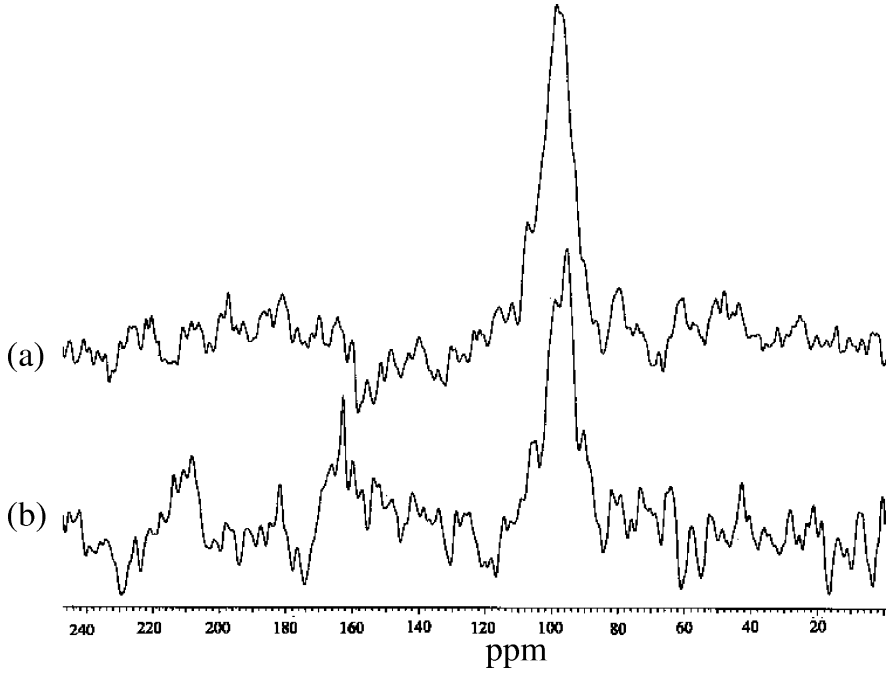

Fig. 6 ^{15}N spectra of aurein in (*a*) hydrated DMPC-d_{54} dispersions and in (*b*) aligned DMPC-d_{54} multilayers with the bilayer normal oriented parallel to the magnetic field; ~52,000 scans were recorded at 30 °C

DMPC bilayers, the ^{2}H and ^{31}P data for both citropin and maculatin also support a peripheral interaction and suggest that these peptides are also mobile in the aqueous phase. All three peptides are largely α-helical when interacting with DMPC bilayers, as shown by earlier CD work with vesicles and oriented multilayers (Marcotte et al. 2003). However, the oriented CD data that suggested that the peptides were aligned with the α-helix long axis ~50° to the bilayer normal were obtained using relatively dry bilayers, which would significantly reduce the mobility of the peptides. Both aurein and citropin are too short to span a lipid bilayer as α-helices, while maculatin, although long enough to adopt a transmembrane orientation, may be flexible due to the proline at residue 15 (Table 1). All three peptides are water soluble and positively charged and are likely to be found in the aqueous phase in neutral (or zwitterionic) membranes. Aurein, citropin and maculatin lyse bacteria but not red blood cells, and the peripheral interaction of these peptides with DMPC, even at very high concentrations (10% molar), may explain their lack of effect on the latter. Since these peptides appear to be in the aqueous phase or interfacial region of the membrane, a "detergent"-like mechanism (Shai 1999) in negatively charged membranes is proposed where the peptides solubilize the membrane, which could result in an isotropic ^{31}P signal as seen with these peptides after long acquisition periods. Since bacterial membranes are negatively charged, the interaction of these peptides with anionic lipids is now being studied.

Conclusions

Peptides from Australian tree frogs adopt an amphipathic α-helical conformation upon interaction with neutral or zwitterionic phospholipid bilayers. Solid-state NMR investigations were carried out on these peptides in both powder and oriented samples using specifically labelled ^{15}N peptides incorporated into DMPC-d_{54} membranes. The ^{2}H and ^{31}P NMR results showed a small effect on the lipid bilayer by these peptides. Both ^{2}H and ^{31}P NMR spectra supported a slowing down of the phospholipids in the presence of the peptides, while the ^{31}P data indicated a disordering of the phospholipid head-groups. The quadrupolar splitting and order parameter of the membrane was reduced by the peptides, suggesting an interfacial location. Solid-state NMR data from specifically labelled peptides suggest that the peptides are highly mobile. ^{15}N spectra of aurein in aligned DMPC-d_{54} bilayers obtained at 0° and 90° orientations and powder dispersions indicate that the peptide is highly mobile and in the aqueous phase in neutral membranes, while the increased disorder of the phospholipid head-groups and acyl chains support an interfacial location for all three peptides. Combining the new data with those of a previous study (Marcotte et al. 2003), we propose that all three peptides are mobile in the aqueous phase in neutral phospholipid bilayers. Future studies of these antibacterial peptides in negatively charged phospholipids are proposed.

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References

Agerberth B, Gunne H, Odedberg J, Kogner P, Boman HG, Gudmundsson GH (1995) FALL-30, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. Proc Natl Acad Sci USA 92:195–199

Bechinger B, Kim Y, Chirlain L, Gesell J, Neumann J-M, Montal M, Tomich J, Zasloff M, Opella S (1991) Orientations of amphipathic helical peptides in membrane bilayers determined by solid-state NMR spectroscopy. J Biomol NMR 1:167–173

Bechinger B, Zasloff M, Opella S (1998) Structure and dynamics of the antibiotic peptide PGLa in membranes by solution and solid-state nuclear magnetic resonance spectroscopy. Biophys J 74:981–987

Boman H (1991) Antibacterial peptides: key components needed in immunity. Cell 65:205–207

Bonev B, Chan W, Bycroft B, Roberts G, Watts A (2000) Interaction of the lantibiotic nisin with mixed lipid bilayers: a ^{31}P and ^{2}H NMR study. Biochemistry 39:11425–11433

Brock TK (1974) Biology of microorganisms. Prentice-Hall, Englewood Cliffs, NJ, USA

Chia B, Carver J, Mulhern T, Bowie J (2000a) Maculatin 1.1, an antimicrobial peptide from the Australian tree frog, *Litoria genimaculata*: solution structure and biological activity. Eur J Biochem 267:1894–1908

Chia BCS, Lam Y-H, Dyall-Smith M, Separovic F, Bowie JH (2000b) A ^{31}P NMR study of the interaction of amphibian antimicrobial peptides with the membranes of live bacteria. Lett Pept Sci 7:151–156

Cornell B, Separovic F, Baldassi A, Smith R (1988) Conformation and orientation of gramicidin A in oriented phospholipid bilayers measured by solid-state carbon-13 NMR. Biophys J 53:67–76

Cross T (1997) Solid-state nuclear magnetic resonance characterization of gramicidin channel structure. Methods Enzymol 289:672–696

de Planque MRR, Greathouse DV, Koeppe RE II, Schafer H, Marsh D, Killian A (1998) Influence of lipid/peptide hydrophobic mismatch on the thickness of diacyl-phosphatidylcholine bilayers. A ^{2}H NMR and ESR study using designed transmembrane α-helical peptides and gramicidin A. Biochemistry 37:9333–9345

Duclohier H, Wroblewski H (2001) Voltage-dependent pore formation and anti-microbial activity by alamethicin and analogues. J Membr Biol 184:1–12

Ehrenstein G, Lecar Q (1977) Electrically gated ionic channels in lipid bilayers. Rev Biophys 10:1–34

Gawrisch K, Barry J, Holte L, Sinnwell T, Bergelson L, Ferretti J (1995) Role of interactions at the lipid-water interface for domain formation. Mol Membr Biol 12:83–88

Gudmundsson G, Agerbeth B, Odedberg J, Bergman T, Olsson B, Salcedo R (1996) The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. Eur J Biochem 238:325–332

Habermann E, Jentsch J (1967) Sequenz analyse des melittins aus den tryptischen und peptischen spaltstucken. Hoppe-Seyler's Z Physiol Chem 348:37–50

Harzer U, Bechinger B (2000) Alignment of lysine-anchored membrane peptides under conditions of hydrophobic mismatch: a CD, ^{15}N and ^{31}P solid-state NMR spectroscopy investigation. Biochemistry 39:13106–13114

He K, Ludtke S, Heller W, Huang H (1996) Mechanism of alamethicin insertion into lipid bilayers. Biophys J 71:2669–2679

Hoffman W, Richter K, Kreil G (1983) A novel peptide designated PYLa and its precursor as predicted from cloned mRNA of *Xenopus laevis* skin. EMBO J 2:711–714

Hong M, Yao XL, Jakes K, Huster D (2002) Investigation of molecular motions by Lee-Goldburg cross-polarization NMR spectroscopy. J Phys Chem B 106:7355–7364

Jacob L, Zasloff M (1994) Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. Ciba Found Symp 186:197–216

Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerrberth B (1998) Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J Biol Chem 273:3718–3724

Mansfield P (1965) Multiple-pulse nuclear magnetic resonance transients in solids. Phys Rev 137:961–974

Marassi F, Ramamoorthy A, Opella S (1997) Complete resolution of the solid-state NMR spectrum of a uniformly ^{15}N-labeled membrane protein in phospholipid bilayers. Proc Natl Acad Sci USA 94:8551–8556

Marassi F, Opella S, Juvvadi P, Merrifield R (1999) Orientations of cecropin A helices in phospholipid bilayers determined by solid-state NMR spectroscopy. Biophys J 77:3152–3155

Marcotte I, Wegener K, Lam Y-H, Chia B, de Planque M, Bowie J, Auger M, Separovic F (2003) Interaction of antimicrobial peptides from Australian amphibians with lipid membranes. Chem Phys Lipids 122:107–120

Nagle J, Tristram-Nagle S (2000) Structure of lipid bilayers. Biochim Biophys Acta 1469:159–195

Oren Z, Hong J, Shai Y (1999) Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell selective activity. Eur J Biochem 341:501–513

Oren Z, Ramesh J, Avrahami D, Suryaprakash N, Shai Y, Jelinek R (2002) Structures and mode of membrane interaction of a short alpha helical lytic peptide and its diastereomer determined by NMR, FTIR and fluorescence spectroscopy. Eur J Biochem 269:3869–3880

Pines A, Gibby M, Waugh J (1973) Proton enhanced NMR of dilute spins in solids. J Chem Phys 59:569–590

Rozek T, Wegener K, Bowie J, Oliver I, Carver J, Wallace J, Tyler M (2000) The antibiotic and anticancer active aurein peptides from the Australian bell frogs *Litoria aurea* and *Litoria rainformis*: the solution structure of aurein 1.2. Eur J Biochem 267:5330–5341

Seelig J (1977) Deuterium magnetic resonance: theory and application to lipid membranes. Q Rev Biophys 10:353–415

Seelig J, Seelig A (1974) The dynamic structure of fatty acyl chains in a phospholipid bilayer measured by deuterium magnetic resonance. Biochemistry 13:4839–4845

Separovic F, Gawrisch K (1996) Effect of unsaturation on the chain order of phosphatidylcholines in a dioleoylphophatidylethanolamine matrix. Biophys J 71:274–282

Shai Y (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462:55–70

Shai Y, Oren Z (2001) From 'carpet' mechanism to *de-novo* designed diastereomeric cell-selective antimicrobial peptides. Peptides 22:1629–1641

Shoji A, Ozaki T, Fujito T, Deguchi K, Ando I (1987) High-resolution ^{15}N NMR study of solid homopolypeptides by the CP MAS method: conformation-dependent ^{15}N chemical shift characteristics of the α-helix and β-sheet forms. Macromolecules 20:2441–2445

Shoji A, Ozaki T, Fujito T, Deguchi K, Ando S, Ando I (1989) Nitrogen-15 NMR chemical shift tensors and conformation of some nitrogen-15-labeled polypeptides in the solid state. Macromolecules 22:2860–2863

Shoji A, Ozaki T, Fujito T, Deguchi K, Ando S, Ando I (1990) ^{15}N chemical shift tensors and conformation of solid polypeptides containing ^{15}N-labeled L-alanine residues by ^{15}N NMR. 2. Secondary structure reflection in σ_{22}. J Am Chem Soc 112:4693–4697

Smith R, Separovic F, Milne T, Whittaker A, Bennett F, Cornell B, Makriyannis A (1994) Structure and orientation of the pore-forming peptide, melittin, in lipid bilayers. J Mol Biol 241:456–466

Steinborner S, Currie G, Bowie J, Wallace J, Tyler M (1998) New caerin peptides from the skin glands of the Australian tree frog *Litoria chloris*. A comparison of the antibiotic activity of maculatin 1.1 and caerin 1.1. J Pept Res 51:121–126

Stone D, Waugh R, Bowie J, Wallace J, Tyler M (1992a) The structure of caerin 1.1, a novel antibiotic from Australian tree frogs. J Chem Soc Chem Commun 1224–1225

Stone D, Waugh R, Bowie J, Wallace J, Tyler M (1992b) Peptides from Australian frogs. Structures of the caerins and caeridin 1 from *Litoria splendida*. J Chem Soc Perkin Trans 1:3173–3178

Wegener K, Wabnitz P, Carver J, Bowie J, Chia B, Wallace J, Tyler M (1999) Host defence peptides from the skin glands of the Australian Blue Mountains tree-frog *Litoria citropa*. The antibacterial citropin 1 peptides. The solution structure of citropin 1.1. Eur J Biochem 265:627–637

Wong H, Bowie J, Carver J (1997) The solution structure and activity of caerin 1.1, an antimicrobial peptide from the Australian green frog, *Litoria splendida*. Eur J Biochem 247:545–557