Author/s:
Sani, MA;Separovic, F

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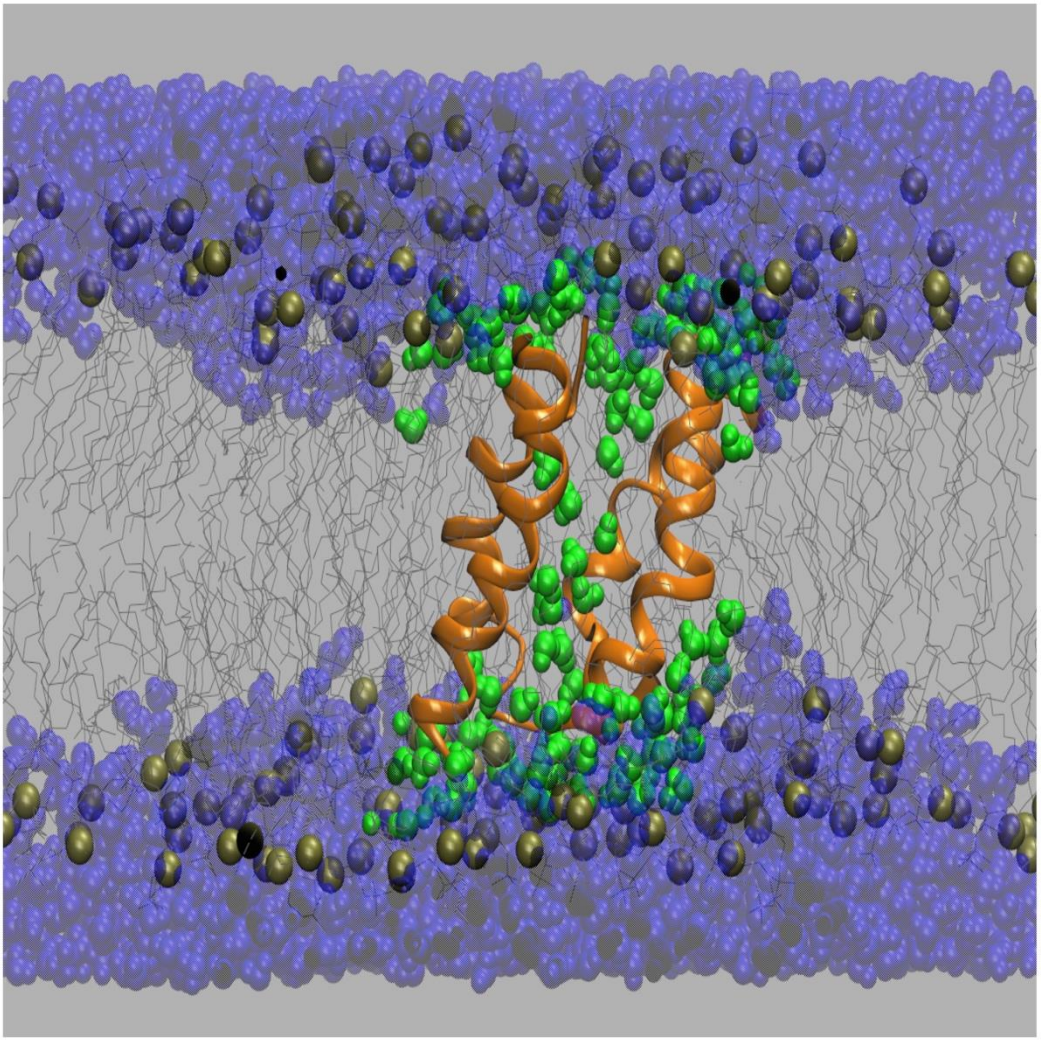
Antimicrobial peptide structure: From model membranes to live cells

Marc-Antoine Sani [a] and Frances Separovic*[b]



[a] *Dr Marc-Antoine Sani*
School of Chemistry, Bio21 Institute
University of Melbourne
Melbourne, VIC 3010 (Australia)

[b] *Professor Frances Separovic*
School of Chemistry, Bio21 Institute
University of Melbourne
Melbourne, VIC 3010 (Australia)
E-mail: fs@unimelb.edu.au

Abstract: The rise in antibiotic resistance has led to a renewed interest in antimicrobial peptides (AMPs) that target membranes. The mode of action of AMPs involves the disruption of the lipid bilayer and leads to growth inhibition and death of the bacteria. However, details at the molecular level of how these peptides kill bacteria and the reasons for the observed differences in selectivity remain unclear. Structural information is crucial for defining the molecular mechanism by which these peptides recognize, self-assemble and interact with a particular lipid membrane. Solid-state NMR is a non-invasive technique that allows study of the structural details of lipid-peptide and peptide-peptide interactions. Following on from studies of antibiotic and lytic peptides, gramicidin A and melittin, respectively, we investigated maculatin 1.1, an AMP from the skin of Australian tree frogs that acts against Gram-positive bacteria. By using perdeuterated phospholipids and specifically labelled peptides, ^{2}H, ^{31}P and {^{31}P}^{15}N REDOR solid-state NMR experiments have been used to localize, maculatin 1.1 in neutral and anionic model membranes. However, the structure, location and activity depend on the composition of the model membrane and current advances in solid-state NMR spectroscopy now allow structure determination of AMPs in live bacteria.

Introduction

Antimicrobial peptides (AMPs) are a class of host defense peptides, typically ten to fifty residues in length, and which usually adopt their active structure only in membrane environments [1]. With the alarming rise in antibiotic resistance [2], there has been increased interest in AMPs and their mode of action. Most AMPs target bacterial cell membranes and act by self-assembling and disrupting the lipid bilayer, which can lead to inhibition of growth or death of the bacteria. The molecular details, however, of how AMPs kill bacteria and how their specificity could be improved remain unclear. Initial studies of these membrane-active peptides in model membranes have provided much information regarding their mechanism of action. In particular, solid-state NMR has given much insight and atomic details of lipid-peptide interactions within phospholipid membranes [3]. Such structural information is essential for defining how these peptides recognize and interact with a particular lipid membrane.

In this Concept article, we discuss our solid-state NMR structure determination of an antibiotic peptide, gramicidin A, and a lytic peptide, melittin from bee venom, in phospholipid bilayers. Then, in greater detail we describe the peptide-lipid interactions of an AMP from the skin secretions of Australian tree frogs, maculatin 1.1, which acts against Gram-positive bacteria [4]. ^{31}P NMR experiments showed more pronounced effect of this cationic peptide on the headgroups of anionic than zwitterionic phospholipid bilayers. Similarly, when using perdeuterated phospholipids, maculatin also led to greater acyl chain perturbation of anionic lipids in mixed phospholipid bilayers [5]. Solid-state NMR experiments were used determine the distance between the ^{15}N/^{13}C labelled peptide and the phosphorous headgroup and acyl chains of the lipid to localize the AMP in neutral and anionic model membranes [unpublished results].

Overall, maculatin adopts a transbilayer orientation and an α-helical structure in model membranes with a stronger interaction with anionic phospholipids. Nonetheless, the structure, location and activity depend on the composition of the model membrane [6]. Such model systems do not approach the complexity of lipid bilayers found in bacteria and eukaryotic cell membranes that AMPs encounter *in vivo*. Current advances in solid-state NMR spectroscopy, however, allow structure determination of AMPs in live bacteria [7] and may lead to better understanding of the structure-function relationships of AMPs.

Membrane structure of an antibiotic peptide

The first clinically tested antibiotic was gramicidin Dubos, an AMP produced by *Bacillus brevis*. Gramicidin Dubos (or gA') [8] is a mixture of four similar peptides of which ~85% is gramicidin A (gA), and was discovered by René Dubos in 1939. Although limited to topical use due to toxicity, gA' helped revive interest in penicillin, which led to the antibiotic era. Gramicidin A acts as an ionophore, which passes monovalent cations, and for decades has served as a model for membrane ion channels. The peptide consists of only 15 residues and has an unusual structure due to its sequence of alternating D- and L- amino acids. The ion channel mechanism was proposed as two helical gA molecules forming a 'pore' in a lipid bilayer membrane. The structure of gA in detergent micelles was determined by solution NMR [9] and postulated as the channel conformation in a cell membrane. Micelles are typically ~2.5 nm in radius whereas are cells of order 2.5-10 μm. To better mimic a cell membrane, phospholipid bilayers and solid-state NMR methods are required to determine gA channel structure [10].

The composition of cell membranes is far more complex than a phospholipid bilayer and can include glycolipids (e.g. ceramides), integral and peripheral membrane proteins (often α-helical transmembrane structure), cholesterol, sphingomyelin and gangliosides. Initial solid-state NMR studies of gA structure were carried out using aligned phosphatidylcholine membranes [10]. Since the NMR signal depends on the orientation of the chemical shielding tensor relative to the magnetic field, the angle of the bond of a specifically labelled atom can be determined in an oriented peptide (Fig. 1). Hence, structural information can be gained from peptides in aligned lipid membranes since the chemical shift (NMR frequency) depends on the angle of the bond to magnetic field [10].

[a] *Dr Marc-Antoine Sani*
School of Chemistry, Bio21 Institute
University of Melbourne
Melbourne, VIC 3010 (Australia)

[b] *Professor Frances Separovic*
School of Chemistry, Bio21 Institute
University of Melbourne
Melbourne, VIC 3010 (Australia)
E-mail: fs@unimelb.edu.au

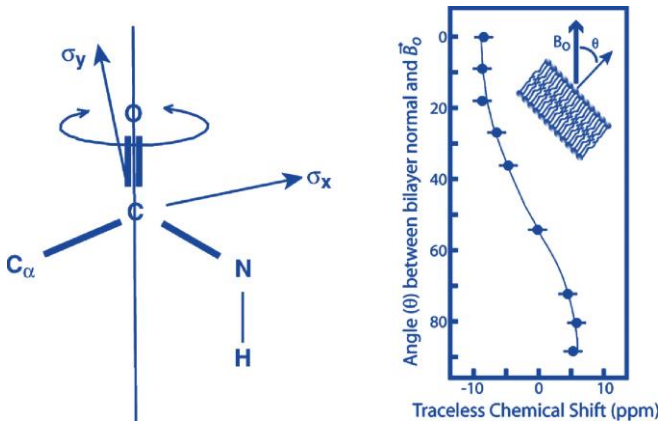


Figure 1. Knowing the magnitude and orientation of the ^{13}C carbonyl shielding tensor, the angle of the C=O bond in a peptide aligned in a phospholipid can be determined and thus the conformation of the peptide [10].

The membrane structure of gA was determined by solid-state NMR using specifically ^{13}C=O labelled peptides [10]. The channel form is a head-to-head, $\beta^{6.3}$ helical dimer (Fig. 2), similar to that found in micelles [11]. Further, ^{13}C solid-state NMR showed that sodium ions bind to the carbonyl of Leu-10 and confirmed that the helix was right-handed [12]. The $\beta^{6.3}$ helical structure forms a stable ion channel, which is preserved in phospholipid membranes of different thickness, from 12 to 18 carbon acyl chains [13]. Solid-state NMR of gA aligned in fluid membranes is able to distinguish between different peptide structures and similar techniques have been applied to structure determination of membrane proteins [14].

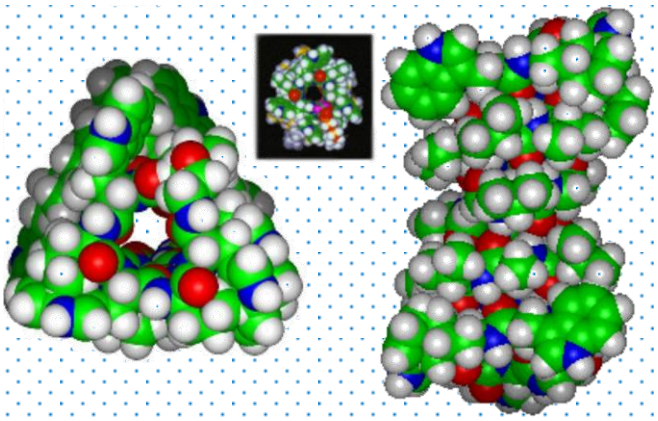


Figure 2. Structure of the gramicidin A dimer as determined from solid-state NMR in a phospholipid bilayer [10, 12].

Structure of a membrane lytic peptide

Melittin is the active peptide in bee venom, whose crystal structure from an aqueous solution was determined as an amphipathic α-helix [15]. The peptide is 26 amino acids in length and acts by lysing cell membranes. The membrane structure of melittin in aligned lipid bilayers was determined by solid-state NMR using ^{13}C specifically labelled peptides [10]. The structure of melittin is dependent on its environment and was found to be kinked in the crystal form and in micelles. In aligned phospholipid bilayers, melittin was α-helical with an angle of 162° about the proline as opposed to ~130° in the crystal structure. The peptide is straighter in a hydrophobic environment or when aligned in a lipid bilayer environment but the structure could be better refined by distance measurements.

Experiments using aligned bilayers provide angular constraints, a but distance measurement by solid-state NMR can be achieved through magic angle spinning (MAS) techniques [16]. The chemical shift anisotropy (CSA) is the dominant interaction for spin ½: depends on field strength (B_0) and orientation of the shielding tensor (σ) in the magnetic field, B_0. The CSA is averaged out by spinning the sample at the magic angle (i.e., MAS) and at a frequency > CSA to gain resolution. Also, the dipolar coupling (DC) involves interaction between two spins and is proportional to the distance and angle of the vector between the two nuclei and B_0. The DC is averaged out by MAS and needs to be reintroduced to perform distance measurements. Both CSA and DC interactions have a $P_2(\cos\ \theta)$ dependence and 3 $\cos^2\theta\ -1 = 0$ at magic angle (54.7°) Thus the structure could be refined using distance measurements by MAS (to increase resolution) and pulse sequences that reintroduce the dipolar coupling and, hence, retrieve distance information. However, a modified CPMG multipulse sequence has been used to measure ^{19}F-^{19}F distance of 6 Å in gramicidin S in aligned phospholipid bilayers [17].

Solid-state NMR of 'powder' or unaligned membranes, where the sample is spun at the magic angle for signal resolution, can be used for structural studies. Using doubly ^{13}C labelled peptides (Fig. 3), the structure of melittin in phospholipid bilayers was refined from MAS distances [18]. Melittin forms toroidal pores in membrane and the peptide structure depends on hydration and the fluidity of the membrane [19].

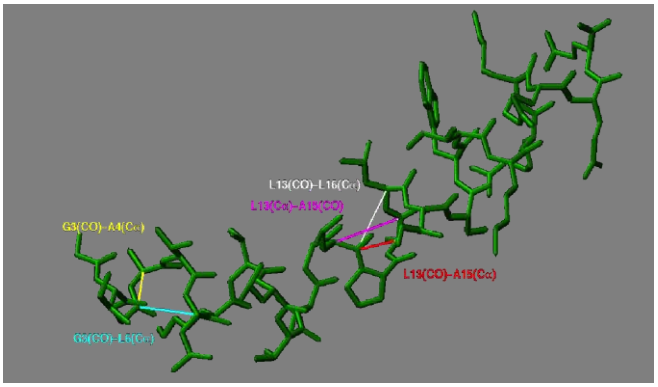


Figure 3. Doubly ^{13}C labelled sites in melittin used for solid-state NMR distance determination in [18].

NMR structural studies of membrane-active AMPs

As well as the solid-state NMR structural determination of these two peptides: (i) gA, which is an antibiotic peptide that acts as an ion channel and has an unusual $\beta^{6.3}$ helix structure in membranes; and (ii) melittin, a peptide toxin that adopts an α-helical structure and lyses cell membranes; the structure of magainin, an antimicrobial α-helical peptide from frog skin was reported [20]. Meanwhile Bowie and co-workers identified a

range of AMPs from Australian tree frogs and we embarked on a study of four of these peptides, whose structure was determined in membrane mimetic environments: (a) aurein 1.2 [21], (b) citropin 1.1 [22], (c) maculatin 1.1 [23], and (d) caerin 1.1 [24]. These amphibian AMPs are of 13-25 amino acids in length, form an amphipathic α-helix in lipid environments and are membrane lytic.

As mentioned earlier, the rise in antibiotic resistance has led to a renewed interest in AMPs. Antibiotic resistance was already identified by Alexander Fleming in the 1940s [25] but is now growing rapidly and new alternatives are needed. The minimum inhibitory concentration (MIC) for the AMP maculatin 1.1 is 8 μg/ml against *S. aureus* (similar to the antibiotic vancomycin) with haemolytic (IH_{50}) values of order 100 μg/ml. These AMPS show some selectivity and are more active against Gram-positive bacteria and cause dye leakage from phospholipid vesicles [25]. A number of models exist for membrane lysis, including the carpet mechanism, barrel-stave and toroidal pore models [1]. We have used a range of techniques to determine the mode of action, such as solid-state NMR, circular dichroism (CD), calorimetry, monolayer studies, surface plasmon resonance, dual polarization interferometry, quartz crystal microbalance, neutron reflectometry and molecular dynamics (MD) simulations. In general, the longer peptides form transmembrane or toroidal pores while the shorter peptides act by a 'carpet'or surface mechanism [27-29]. However, the mechanism (Fig. 4) depends on the type of lipid and peptide concentration.

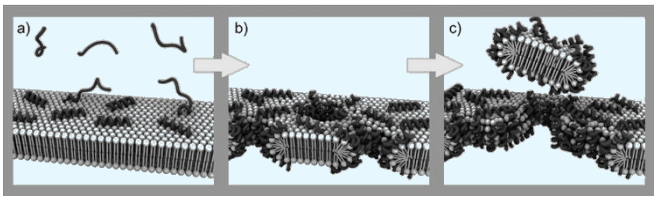


Figure 4. (a) Unstructured peptides assemble on surface of membrane as a-helices; with increasing concentration, the peptides (b) form toroidal pores, and (c) eventually disintegrate the membrane. Image adapted from [15].

Monolayer studies showed that these AMPs insert into 'bacterial' membranes, i.e., anionic phospholipid monolayers, as α-helical peptides but were immiscible in zwitterionic monolayers [30]. Overall, these AMPs are located in aqueous phase of 'neutral' membranes but have a stronger interaction with negatively charged lipids. In addition, their activity depends on lipid content with the longer peptides forming pores while the shorter peptides disintegrate membranes [31].

Structure of maculatin 1.1 in model membranes

Following mutation studies [32], we have concentrated on maculatin 1.1 as an antibiotic alternative and investigated how lipid composition plays a role in peptide selectivity and activity. Maculatin 1.1 (Mac1) has 21 residues with sequence:

$^{+}$**H$_3$N**-GLFGVLA**K**VAAHVVPAIA**E**HF-NH$_2$ cationic (+1)

Interestingly, Mac1 is more lytic against neutral rather than negatively charged phospholipid vesicles but, in a competitive lipid environment with both types of vesicles, Mac1 barely interacts with neutral bilayers in the presence of the latter [33]. So, although Mac1 inserts into neutral bilayers, the affinity for anionic membranes is stronger which would translate to a more targeted interaction with bacterial membranes.

We used solution NMR and CD spectroscopy to investigate the peptide structure and location of Mac1 in different membrane systems. Using ^{15}N labeled peptide, Mac1 is unstructured in buffer and α-helical in zwitterionic and anionic micelles as well as bicelles. Solvent exposure was determined using Gd^{3+}(DTPA) which indicated a transbilayer orientation in bicelles, in agreement with our CD results [34], which indicate a hydrophobic match for Mac1 and DMPC, a 14 carbon chain length phospholipid. Using deuterated phospholipids, ^{2}H and ^{31}P solid-state NMR data show a greater effect on the anionic lipid. The peptide structure and location in phospholipid bilayers was confirmed using heteronuclear solid-state NMR, Rotational Echo Double Resonance (REDOR), distance measurements [16]. REDOR was used to measure intra- and intermolecular distances using specifically labelled Mac1 peptides. Since MAS averages the dipole coupling to zero over one rotor period, selective 180º pulses are applied to reintroduce heteronuclear dipole coupling and as a result, the signal is attenuated based on the distance between the nuclei. REDOR distance measurements in ^{13}C-^{15}N Mac1 and {^{31}P} ^{13}C/^{15}N REDOR are consistent with an α-helical peptide in a transmembrane configuration in phospholipid bilayers (Fig. 5) and consistent with MD simulations [35].

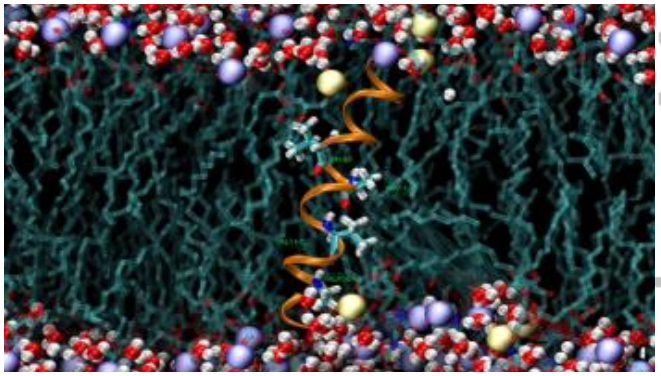


Figure 5. A configuration of a single Mac1 peptide in a mixed phospholipid bilayer.

NMR of Mac1 in live bacteria

Since the structure of AMPs is highly dependent on the membrane environment, then rather than NMR studies in more complex phospholipid bilayers, we have embarked on studies in live bacteria [7, 36]. Recent developments in dynamic nuclear polarization (DNP) [37] have led to tremendous enhancement in NMR by factors of 10^2 to 10^3, and the possibility of studying AMPs at physiological concentrations in live bacteria. By adding the spin label amino acid, TOAC, to the N-terminus of Mac1, DNP enhancement of specifically ^{13}C and ^{15}N labelled Mac1 in bacteria was observed at 8 kHz MAS speed at 110 K. In addition, DNP-enhanced ^{31}P signals of membrane phospholipids in *E. coli* indicated that the TOAC-Mac1 was located in the bacterial membrane. Future investigations will be coupled with MD results [35] to determine label positions for DNP-NMR and lead to in-cell NMR structure determination of AMPs.

Conclusions

Studies of AMPs show that the peptide structure dependent on lipid composition of model membranes. Thus, care is required when in choosing membrane mimetics for structural studies. Developments in solid-state NMR have led to structure of AMPs being determined in model membranes and progress to live cells studies. ^{31}P and ^{2}H solid-state NMR showed more pronounced effect of these cationic peptides on the headgroups and acyl chains of anionic rather than zwitterionic phospholipid bilayers which mimic bacterial and eukaryotic membranes, respectively. REDOR NMR experiments have been used to probe the penetration depth of AMPs by determination of the distance between the $^{15}N/^{13}C$ labelled peptide and the phosphorous headgroup and the acyl chains of the lipid. The AMP maculatin 1.1. adopts an α-helical structure and transbilayer orientation in mixed phospholipid membranes and solid-state NMR structural studies in live bacteria are underway. In particular, how lipid composition modulates the mechanism of AMP self-assembly is a crucial piece of the puzzle still to be elucidated**.** This challenge may well be addressed by in-cell DNP-NMR studies using recombinant expression of labelled AMPs [38] and bacteria that have been genetically engineered to have different membrane lipid compositions [39].

Acknowledgements

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Keywords: antimicrobial peptides • solid-state NMR • in-cell NMR • membrane structure • phospholipid bilayers

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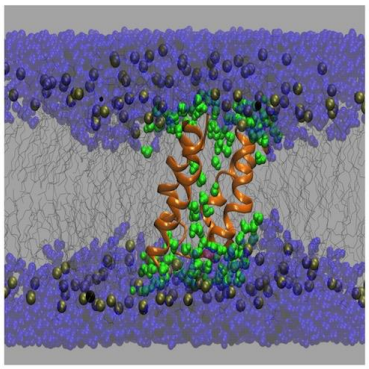
Entry for the Table of Contents (Please choose one layout)

Layout 2:

CONCEPT

Developments in biological solid-sate NMR have led to structure determination of membrane-active peptides in cell membranes.



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