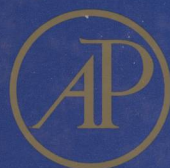


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VOLUME 65

Edited by

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*Royal Society of Chemistry
Burlington House
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VOLUME **65**

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PREFACE

It is my pleasure to introduce Volume 65 of the Annual Reports on NMR Spectroscopy. Readers of this series will be familiar with the wide range of topics covered in each volume. The present one is an exception, in that almost all of the areas covered have a biological flavour. It begins with an account of 'NMR of Antimicrobial Peptides' by E. F. Haney and H. J. Vogel; this is followed by an account from M. Mizuguchi, T. Aizawa, K. Kawano and M. Demura on 'NMR Studies of Protein Folding: Folding Studies of Calcium-Binding Lysozyme and α -Lactalbumin'; 'Applications of the NOE in Molecular Biology' is covered by M. P. Williamson; T. Gullion reports on 'Recent Applications of REDOR to Biological Problems'; 'Electrophoretic NMR-Ions, Molecules, Mixtures, Pores and Complexes' are covered by P. C. Griffiths; finally, S. Ramadan and C. E. Mountford provide an account of 'Two-Dimensional NMR on Biopsy and In Vivo'.

My sincere thanks go to all of these reporters for their timely and well presented contributions.

G. A. Webb
Royal Society of Chemistry, Burlington House, Piccadilly, London, UK
February 2008

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NMR of Antimicrobial Peptides

Evan F. Haney and **Hans J. Vogel**

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Abstract

The continuing increase in the resistance of pathogenic bacteria to conventional antibiotics has led to the emergence of new strains that are often referred to as 'superbugs'. There is a serious need for new anti-infective therapies as clinicians can no longer effectively combat these bacterial pathogens. In the search for new and effective antibiotic compounds, antimicrobial peptides, which are part of a larger group of host defence peptides and proteins, have emerged as a potential class of agents that may stem the tide of antibiotic resistance. However, the mechanism of action of antimicrobial peptides is currently poorly understood. Widely different and complex

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models for the microbicidal action of antimicrobial peptides have been proposed. Consequently, in order to optimize the design of new synthetic antimicrobial peptides, each peptide must be examined on its own to determine which factors are most responsible for its bactericidal or bacteriostatic activity. Nuclear magnetic resonance (NMR) spectroscopy has become the tool of choice for determining the structures of the antimicrobial peptides and to characterize their interactions with their initial target, the negatively charged bacterial membrane. Moreover, interactions with zwitterionic eukaryotic membranes are also surveyed in an attempt to avoid host cytotoxicity of these peptides. Standard solution-state NMR techniques are now commonly used to determine the high-resolution structure of an antimicrobial peptide in aqueous solution, in detergent micelles or in membrane mimetic organic solvents. Solid-state NMR is increasingly being used to examine the orientation of the peptide in phospholipid bilayers as well as the oligomeric state of the peptide; it can also be used to characterize the changes in biological membranes that are induced upon peptide binding. Through the incorporation of ^{15}N , ^{13}C and ^2H nuclei into antimicrobial peptides by recombinant fusion-protein expression techniques, as well as advances in NMR technology and experimental procedures, antimicrobial peptides can now be studied in large membrane mimetic vesicles which more closely resemble biological membrane bilayers *in vivo*. This review paper will discuss many of the solution and solid-state NMR experiments that have been used to characterize antimicrobial peptides and that have provided researchers with unique insights into the diverse mechanisms of action of this class of biomolecules.

Key Words: Antimicrobial peptides, Nuclear magnetic resonance spectroscopy, Mechanism of antimicrobial activity, Detergent micelle, Peptide structure determination, Membrane perturbation.

1. INTRODUCTION

Small peptides with the ability to selectively kill bacterial cells were reported as early as 1981 when the primary structure of insect cecropin was published by Hans Boman and co-workers.¹ Cecropins were identified as important components of the insect immune system and it was assumed that this type of mechanism would not be present in the more sophisticated immune system of higher animals. However, research into antimicrobial peptides exploded when the first peptide from an animal was discovered on the skin of the African clawed frog, *Xenopus leavis*.² This peptide was discovered virtually by accident when Michael Zasloff was working on oocytes harvested from female frogs. Dr. Zasloff used a non-sterile surgical technique to harvest the oocytes from female frogs, sutured the wounds and then placed the animals back into a holding tank. In spite of the unsterile surgical procedures and the microbially rich environment present in the holding tanks, these frogs rarely developed infections and the wound healed normally. Zasloff hypothesized that the skin of the frogs had 'sterilizing' activity

and proceeded to isolate a small antimicrobial peptide from the skin of the frog that he named magainin.²

Since that time, antimicrobial peptides have been identified in insects,³ plants,⁴ animals⁵ and humans.⁶ Even bacterial cells have been shown to produce peptides with bactericidal activity⁷ which gives them an evolutionary advantage against competing microorganisms in the same environment. Antimicrobial peptides are now recognized as an important part of the innate immune system and often are the first line of defence against invading pathogens. Research into antimicrobial peptides is fuelled by the increasing resistance of clinically relevant bacterial strains to commonly used antibiotics.⁸ This creates a need to develop new bactericidal compounds to replace the ineffective conventional antibiotics. Antimicrobial peptides with different amino acid sequences are continually being discovered and synthetically altered in an attempt to generate more powerful antibacterial molecules and to understand the underlying mechanism of activity of this class of peptides. There are a few antimicrobial peptide databases that have been set up to catalogue the ever increasing number of peptide sequences with antibacterial effects. One of these was established in 2004 by Guangshun Wang and co-workers at the University of Nebraska⁹ and it now contains information on over 700 peptide sequences with antibacterial, antifungal, antiviral and anticancer activities (<http://aps.unmc.edu/AP/main.php>). Another database was established even earlier by the research group of Allesandro Tossi at the University of Trieste in Italy (<http://www.bbcm.units.it/~tossi/amsdb.html>) with the same goal of cataloguing these antibacterial molecules as they are reported in the literature.

Antimicrobial peptides show a tremendous amount of variation in their primary sequences and structures. Typically, naturally occurring antimicrobial peptides range in size from 12 to 60 residues; however, synthetic peptides as short as six residues have been shown to have significant antimicrobial activity.¹⁰ Most antimicrobial peptides have a net positive charge owing to a preponderance of lysine and arginine residues. The cationic nature of these peptides is believed to be responsible for the selectivity of antimicrobial peptides for bacterial membranes (discussed below). Antimicrobial peptides also possess a high proportion of hydrophobic residues such as phenylalanine, leucine, valine and alanine.¹¹ Tryptophan residues are found in unusually high proportions in some classes of antimicrobial peptides¹² and the fact that they have a preference for the interfacial region of lipid bilayers¹³ also has implications for their mechanism of action.

Numerous structures of antimicrobial peptides are reported in the literature. They typically adopt amphipathic conformations with one face containing the cationic amino acids and the opposite face possessing the hydrophobic amino acids. We have previously attempted to classify antimicrobial peptides based on their structural characteristics and their amino acid composition.¹⁴ Antimicrobial peptides can be separated into five classes: amphipathic α -helices, β -sheet peptides, cysteine-rich peptides, peptides with an unusually high content of a common amino acid and peptides with uncommon and/or chemically-modified amino acids.

Structural characterization of antimicrobial peptides is a vital step in understanding the underlying mechanism of antimicrobial activity of this class of

compounds. X-ray crystallography and Nuclear Magnetic Resonance (NMR) Spectroscopy are the two most important techniques for high resolution structure determination of biomolecules, such as proteins. However, in the case of antimicrobial peptides, the use of NMR has been much more prevalent than X-ray crystallography, as evidenced by the fact that the overwhelming majority of the high resolution structures of antimicrobial peptides deposited in the Protein Data Bank were solved using various NMR methods. Obtaining high quality crystals of antimicrobial peptides in a lipid environment is extremely difficult because of their small size, intrinsic flexibility and the inherent difficulties associated with crystallizing membrane proteins. On the other hand, NMR spectroscopy has proven to be a versatile tool for studying antimicrobial peptides. Recent reviews have examined the structural characterization of antimicrobial peptides and how they interact with phospholipids.^{12,14-19}

This chapter is not meant to be a comprehensive review of all of the NMR studies reported for antimicrobial peptides, nor will it delve into detail regarding the theoretical considerations underlying the experimental techniques used. Instead, this review will examine the type of information that can be obtained from NMR spectroscopy when studying antimicrobial peptides and how this information can be applied to decipher their mechanisms of action when killing bacterial cells. Additionally, we will discuss some of the limitations of the techniques used and examine some key considerations when analyzing the results.

2. MECHANISM OF ACTION

Owing to the large diversity in sequence and structures, the mechanisms of action of antimicrobial peptides are also varied and diverse. Many antimicrobial peptides are believed to interact with the surface of bacterial cells and induce membrane perturbations. Peptides are thought to induce structural changes in the bacterial membrane that lead to the formation of pores or the solubilisation of the membrane, both of which can lead to the disruption of electrochemical gradients and the release of intracellular components to the environment. The most popular pore forming models are the barrel-stave model and the toroidal pore model in which the antimicrobial peptides oligomerize to form transmembrane pores. In the barrel-stave model (Figure 1A), the pore is completely lined by peptides with the hydrophobic face of the amphipathic molecule interacting with the acyl chains of the bilayer.²⁰ This model was originally proposed to explain the antimicrobial activity of alamethicin²¹ and the structure of this pore was recently solved using X-ray diffraction studies and found to contain eight alamethicin helices per transmembrane pore.²² However, the barrel-stave model has proven to be relatively ineffective at explaining the antimicrobial activity of the myriads of other antimicrobial peptides. It may well be that the bacterial anionic alamethicin peptide is simply not a good model for the cationic antimicrobial peptides found in eukaryotes.

In the toroidal pore model (Figure 1C), the antimicrobial peptides induce a curvature strain on the leaflets of the bilayer until the two sides of the bilayer fuse to form a peptide/lipid lined pore.²³ This mechanism has been invoked to explain

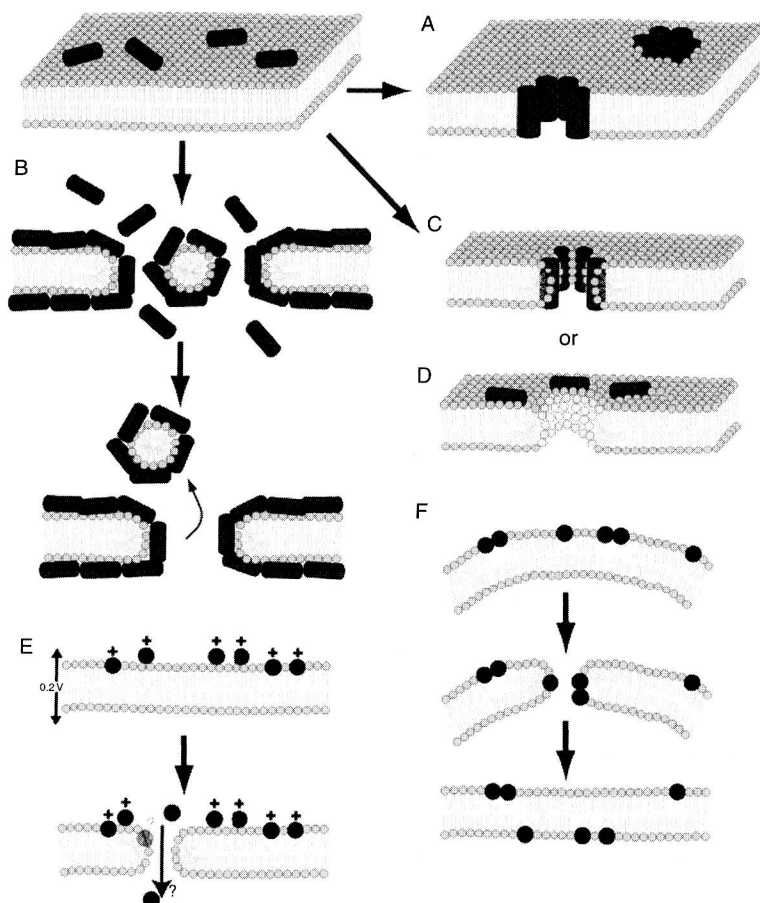


Figure 1 Proposed models for the mechanisms of antimicrobial activity of antimicrobial peptides. Following aggregation of the antimicrobial peptide on the surface of the bilayer, the peptide can disrupt the bilayer through; the barrel-stave model (A), the carpet mechanism (B), the toroidal pore model (C), the in plane diffusion model (D), the electroporation model (E) or the sinking raft model (F). (Figure adapted from Ref. 12, with permission).

the antimicrobial activity of a number of antimicrobial peptides including magainin 2,²⁴ piscidin²⁵ and LL-37.²⁶ A mechanism similar to the toroidal pore model is the in-plane diffusion model²⁷ (Figure 1D) which is applicable to peptides that induce local membrane perturbations close to the region where the peptide binds and causes the formation of a lipid lined pore that is stabilized by peptides bound to the surface of the bilayer. Experimental evidence for this type of mechanism is limited but molecular dynamics simulations of the magainin analog MG-H2 have demonstrated that a pore forms spontaneously when this peptide is mixed with DPPC bilayers. Of significant importance is that only one peptide is found inside the pore while the remainder stabilize the opening by binding to the rim of the pore.²⁸

The carpet model of antimicrobial activity (Figure 1B) is also a popular mechanism to explain the antimicrobial activity of many antimicrobial peptides.²⁰ This

model suggests that the peptides accumulate on the surface of the bacterial membrane and once an effective concentration is reached, they induce the micellization of the membrane and essentially dissolve part of the phospholipid bilayer, leading to cell death.

Other mechanisms for antimicrobial activity have been proposed and although they receive less attention than those already mentioned, they are nonetheless relevant when discussing the possible modes of action of this class of antibacterial molecules. The electroporation model (Figure 1E) explains the formation of transient membrane pores as a result of the electric potential that is created across a membrane due to the accumulation of a large number of cationic peptides on one side of the bilayer.^{29,30} The sinking raft model (Figure 1F) also advocates the formation of transient pores in the membrane. In this model, the peptides aggregate on the outer leaflet of the membrane which creates a mass imbalance across the bilayer. This induces changes in the local membrane curvature around this peptide oligomer and these peptides eventually pass through the membrane and redistribute themselves along both sides of the bilayer.³¹ While passing through the membrane these peptide bundles induce transient pores that have the same effect as the pores mentioned earlier.

Some antimicrobial peptides have relatively little effect on membrane stability but instead pass through the phospholipid bilayer and exert their antimicrobial effect on intracellular targets.¹¹ The short proline-rich family of antimicrobial peptides are an example of this type of peptide as they are thought to inhibit intracellular protein synthesis and/or protein folding. Most of the antimicrobial peptides in this family are produced by insects; however, there are also several mammalian examples of proline-rich antimicrobial peptides.³² Proline residues in antimicrobial peptides often have distinct effects on the ability of these antibiotic molecules to disrupt the integrity of a bacterial membrane. Inserting a proline residue into an α -helical antimicrobial peptide with known membrane destabilizing properties decreases the membrane permeabilization ability of that peptide.³³ The opposite situation has also been observed for buforin II, a potent 21-residue antimicrobial peptide derived from a naturally occurring peptide found in the stomach of the Asian toad.³⁴ Buforin II is an α -helical antimicrobial peptide with a proline hinge that is capable of quickly killing bacterial cells but it has no effect on the integrity of the plasma membrane.³⁵ Interestingly, buforin II analogs that lack this proline hinge turn into membrane destabilizing peptides indicating that the proline residue is essential for the translocation mechanism across the membrane.³⁵

Other antimicrobial peptides with different structural motifs also appear to act on intracellular targets within bacterial cells with no appreciable effect on the integrity of the phospholipid bilayer. Polyphemusin is a β -hairpin peptide that displays antimicrobial activity *in vitro* and causes lipid flip-flop but it does not induce significant vesicle leakage.³⁶ In fact, polyphemusin accumulates in the cytoplasm of *E. coli* cells without any measurable damage to the integrity of the bacterial membrane.³⁷ The proposed mechanism used by polyphemusin to cross the bacterial membrane is similar to the sinking raft model except that the peptide induces negative curvature strain on the bilayer and then translocates across the

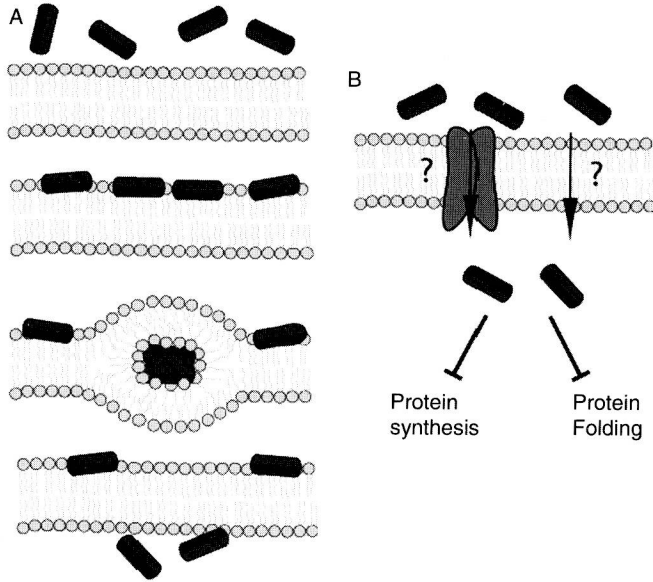


Figure 2 Proposed non-bilayer perturbing mechanisms of membrane translocation of antimicrobial peptides. Antimicrobial peptides may pass through the membrane through a non-bilayer intermediate like that proposed for polyphemusin³⁶ (A). Antimicrobial peptides could also reach the cytoplasm through integral membrane proteins or through some other unknown mechanism (B).

membrane through a non-bilayer intermediate without disrupting the integrity of the plasma membrane (Figure 2A).

Certain antimicrobial peptides do not interact directly with the membrane but instead they cross the bacterial membrane through an integral membrane receptor or by some other unknown mechanism (Figure 2B). For example, bacteriocins are a class of antimicrobial peptides that are ribosomally synthesized by bacterial cells as a means of out-competing other species in the local environment for nutrients and resources. Microcin E492 is a small bacteriocin produced by *Klebsiella pneumoniae* with activity against other Enterobacteriaceae species.^{38–40} The mechanism of action of this peptide is not completely understood but there are various bacterial membrane receptors that have been implicated in its activity. Microcin E492 is recognized by the outer membrane receptors FepA, Fiu and Cir⁴¹ which is likely due to a post-translational modification of microcin that causes it to mimic a catechol-type siderophore.³⁹ Additional evidence indicates that microcin E492 inserts into the inner membrane of bacterial cells and induces the formation of pores, leading to membrane depolarization and increased permeability.^{42,43} A recent mutagenesis study also found that an inner membrane protein complex responsible for mannose uptake is essential for the bactericidal activity of this antimicrobial peptide.⁴⁴ The mannose phosphotransferase system has also been implicated as a receptor for lactococcin A, lactococcin B and other pediocin-like bacteriocins.⁴⁵

Bacteriocins are not the only antimicrobial peptides that take advantage of transmembrane receptor proteins to transport across the bacterial membrane.

The interaction between *E. coli* cells and the proline-rich antimicrobial peptide Bac-7 (1–35) was examined and peptide translocation appears to be mediated by an inner membrane receptor encoded by the *sbmA* gene.⁴⁶ The *sbmA* gene encodes a 407 residue inner membrane protein in Gram-negative bacteria⁴⁷ and is known to be important for the uptake of other microcins into *E. coli* cells.^{48,49} In this particular study, *E. coli* mutants with a point mutation in the *sbmA* gene or an *sbmA* knockout were much less susceptible to the antimicrobial activity of Bac-7; in contrast, overexpression of the *sbmA* gene made these cells more sensitive to the effects of this peptide.⁴⁶ Although the exact mechanism through which the *sbmA* gene product translocates this proline-rich antimicrobial peptide is presently unclear, the role of transmembrane proteins in bacterial cells as a means of crossing the phospholipid bilayer needs to be considered when discussing which mechanisms of action antimicrobial peptides rely on to exert their antibiotic effects. Clearly, specific interactions mediated by bacterial membrane receptors can be responsible for membrane translocation while the other mechanisms of antimicrobial activity are considered non-specific in their mode of action.

Finally, we should point out that there is an increasing amount of evidence that points to intracellular targets for those antimicrobial peptides that do not cause serious destabilization of the phospholipid bilayer of bacterial cells. Buforin II is thought to act by spontaneously crossing the cell membrane and enter the cytoplasm of the cell where it is free to bind the bacterial DNA and RNA and consequently inhibit normal cellular functioning.⁵⁰ Direct evidence of an antimicrobial peptide binding to DNA has been observed for indolicidin, a peptide found in bovine neutrophils, where peptide binding was confirmed through Trp fluorescence quenching upon interaction with DNA and the observation that indolicidin inhibits DNA migration in agarose gels.⁵¹ Additionally, an all D-amino acid form of the proline-rich antimicrobial peptide apidaecin is inactive,⁵² suggesting that this peptide binds to an intracellular target in a stereospecific manner as opposed to the non-specific interactions with the bacterial membrane described earlier.

Because of the widely divergent proposed models of antimicrobial activity, numerous questions need to be answered to elucidate the antimicrobial mechanism for a given peptide. What is the structure of this molecule in aqueous solution? Does the conformation of the peptide change in the presence of lipids? Is there a preference for a charged lipid species versus a zwitterionic one? Is the peptide oriented along the surface of the bilayer or does it adopt a transmembrane conformation? How deeply does the peptide insert itself into the hydrophobic core of a bilayer? Does the peptide affect the structure and organization of bilayers? Answers to all these questions, and many more, can be obtained through the use of NMR experiments using both solution and solid-state NMR techniques.

3. SOLUTION-STATE NMR

The small size of antimicrobial peptides makes them attractive candidates for structural studies using solution-state NMR. The experimental approach for determining the solution structure of this class of peptides is well established