

Cationic membrane peptides: atomic-level insight of structure–activity relationships from solid-state NMR

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Abstract Many membrane-active peptides, such as cationic cell-penetrating peptides (CPPs) and antimicrobial peptides (AMPs), conduct their biological functions by interacting with the cell membrane. The interactions of charged residues with lipids and water facilitate membrane insertion, translocation or disruption of these highly hydrophobic species. In this review, we will summarize high-resolution structural and dynamic findings towards the understanding of the structure–activity relationship of lipid membrane-bound CPPs and AMPs, as examples of the current development of solid-state NMR (SSNMR) techniques for studying membrane peptides. We will present the most recent atomic-resolution structure of the guanidinium-phosphate complex, as constrained from experimentally measured site-specific distances. These SSNMR results will be valuable specifically for understanding the intracellular translocation pathway of CPPs and antimicrobial mechanism of AMPs, and more generally broaden our insight into how cationic macromolecules interact with and cross the lipid membrane.

Keywords Cationic membrane peptide · Cell-penetrating peptides · Antimicrobial peptides · Guanidinium-phosphate complex · Solid-state NMR

Abbreviations

AMP	Antimicrobial peptide
CPP	Cell-penetrating peptide
DARR	Dipolar-assisted rotational resonance
DIPSHIFT	Dipolar-chemical-shift correlation
DNP	Dynamic nuclear polarization
H-bond	Hydrogen bond
HETCOR	Heteronuclear correlation
HNP-1	Human neutrophil peptide-1
LPS	Lipopolysaccharide
MAS	Magic angle spinning
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PG-1	Protegrin-1
POPC	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
POPE	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol
PRE	Paramagnetic relaxation enhancement
PTD	Protein transduction domain
REDOR	Rotational-echo double-resonance

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Introduction

A large number of functionally important proteins and peptides carry out their functions in the cellular membrane,

among which cationic cell-penetrating peptides (CPPs) and antimicrobial peptides (AMPs) have recently attracted much attention. Cellular internalization of bioactive agents, such as therapeutics, imaging agents and reporter molecules, garners significant biomedical and pharmaceutical interest, because these molecules must bind to intracellular targets to function. However, the impermeable nature of the plasma membrane inhibits cellular entry of hydrophilic molecules that are relatively large. CPPs, a category of small peptides usually rich in arginine and lysine residues, have remarkable cellular uptake ability to deliver electrostatically or covalently bound bioactive agents into cells in culture and preclinical models in vivo (Zhang and Smith 2005; Schug and Lindner 2005; Fischer et al. 2005). Another important category of cationic membrane peptides is AMPs. They are the important components of the innate immune system of many animals and plants (Zasloff 2002). They kill a broad spectrum of microbes including bacteria and fungi by destroying their cell membranes (Matsuzaki 1999). This rapid and general mechanism of action makes AMPs appealing alternatives to conventional antibiotics, most of which are rendered ineffective due to the increased incidence of antibiotic-resistant microbes.

CPPs and AMPs share common characteristics. Structurally, they have a highly charged character, a relatively small size (<35 amino acids) and are lipid-bound. Functionally, the similar membrane insertion dictates the non-invasive cellular internalization potential of CPPs as drug transporters, and is responsible for the antimicrobial efficiency and selectivity of AMPs. The mechanism addressing how these cationic peptides overcome the low-dielectric hydrophobic interior of the cell membrane and insert into the lipid bilayer remains unclear, limiting their therapeutic applications and presenting challenges to the design of more efficient alternatives (Chan et al. 2006; von Heijne 2006). This question has remained largely unanswered due to difficulty of characterizing the structures of these complex membrane systems at the atomic-level. CPPs and AMPs are similar in structural motif of amino acid composition,

but differ in biological activities within the lipid membrane. This begs the question, what is the underlying mechanism that distinguishes the two structurally similar but functionally different species?

To address these questions, SSNMR presents the most facile techniques, because it can probe the high-resolution structures of insoluble and noncrystalline membrane proteins that are difficult to analyze by traditional X-ray crystallography and solution NMR techniques (McDermott 2009; Tycko 2011; Hong et al. 2012b). Use of isotopic labeling, magic angle spinning (MAS) and multi-pulse techniques have made high-resolution NMR feasible in the solid state (De Paëpe 2012; Hong 1999; Rienstra et al. 2000). In recent years, it has gained much importance as a versatile tool to elucidate the structure, dynamics and functions of membrane-bound proteins, protein complexes, sedimented proteins, amyloid fibrils and more (Hong et al. 2012b; Tycko 2011; Bertini et al. 2011; Renault et al. 2011; Hong and Su 2011).

In the past few years, we have extensively investigated many CPPs and AMPs to elucidate the structural basis of how these cationic macromolecules interact with membrane lipids and water (Hong and Su 2011; Hong 2006) (Table 1). The two CPPs studied in our research, TAT and penetratin, are the first two discovered and also most frequently applied CPPs (Hong and Su 2011). TAT peptide contains the protein transduction domain (PTD) of the trans-activator of transcription (TAT protein) of HIV-1 virus (Green and Loewenstein 1988; Frankel and Pabo 1988), and penetratin is derived from the *Drosophila* homeodomain protein (Derossi et al. 1994). Two examples of AMPs in our recent studies include Protegrin-1 (PG-1) (Tang and Hong 2009) and human neutrophil peptide-1 (HNP-1) (Zhang et al. 2010a, b). PG-1 is the representative of many β -sheet AMPs in its disulfide-linked structure and Arg-rich sequence. HNP-1 belongs to the α -defensin family of antimicrobial peptides, and is the mediator of the host innate immune response. It is larger in size than PG-1 and contains different conformational domains.

Table 1 Examples of cationic membrane-active peptides showing guanidinium-phosphate interactions. Cationic residues (Arg and Lys) are underlined in peptide sequences

Category	Peptide	Origins	Sequence	Conformation	$r(C_{\epsilon}-P)$
CPP	TAT	The basic domain of TAT protein of HIV-1 virus	<u>GRKRRRQRRRPPQ</u>	Random coil	R8: 4.1 Å
	Penetratin	The 3rd helix of Antennapedia Homeodomain	<u>RQIKIWFQNRRMKWKK</u>	Coil-like	R10: 4.3 Å
AMP	PG-1	Porcine leukocytes	<u>RGGRLCYCRRRFCVCGR</u>	β -Hairpin	R11: 4.0 Å
	HNP-1	Human α -defensin	<u>ACYCRIPACIAGERRY</u> <u>GTCIYQGRLWAFCC</u>	β -Strand-rich	R25: 4.0 Å

Refer to the main text for detailed references. All conformations were determined in phospholipid bilayers at physiological temperature. $r(C_{\epsilon}-P)$ refers to the atomic distance between guanidinium C_{ϵ} of the specified arginine and the phosphorus atom of the lipid headgroup

In this review, we will first address the crucial interactions of cationic residues, Arg and Lys, with lipids and water, which is widely recognized to play important roles in membrane protein function. Experimental evidences verifying the existence of these important interactions will be present. We'll then have two separate sections of CPPs and AMPs as examples to demonstrate the indispensable role of solid-state NMR for elucidating the structure and dynamics of membrane-bound macromolecules. In each section, we will mainly focus on elucidating how these SSNMR findings give specific insights about the corresponding mechanism of action. Finally, the structural basis distinguishing these two categories of membrane-active peptides will be discussed.

Peptide-lipid and peptide-water interactions

An ultimate goal of membrane protein studies is to understand the underlying molecular mechanisms of their biological functions. One important facet is to address the functional contributions of intermolecular interactions at an atomic or molecular level. Compared with the relatively well established biophysical and biochemical tools for analyzing structures and interactions of globular proteins, the study of membrane proteins is still relatively nascent. This is due to the extraordinary complexity of the membrane environment for most structural analysis methods. For example, the translocation efficiency and kinetics of CPPs and the antimicrobial potency of AMPs have been extensively studied, however, the atomic-level structural basis of how they interact with lipids to conduct their biological functions remains mysterious and unclear. Here, we will show the discovery of mechanistically essential peptide-lipid and peptide-water interactions in our recent SSNMR studies.

Arg guanidinium-phosphate and Lys ammonium-phosphate interactions

Charge interactions between cationic residues and lipids are essential for the biological function of many membrane peptides and proteins, for example, cellular translocation of CPPs, membrane disruption of AMPs, and gating of voltage-sensing K^+ channels (Freites et al. 2005; Tang and Hong 2009; Schmidt et al. 2009). The highly basic Arg guanidinium and Lys ammonium groups remain protonated under physiological pH conditions, and thus can function as hydrogen bond (H-bond) donors in various protein-protein and protein-lipid interactions. The guanidinium-phosphate salt bridge is involved in many biological functions. As early as 1991, this interaction was proposed by Frankel et al. to explain site-specific binding to RNA

(Calnan et al. 1991). As a highly stable cation in aqueous solutions, the guanidinium group accounts for most of Arg's noncovalent bonding with anionic groups such as phosphates, sulfates and carboxylates (Sanchez-Quesada et al. 1996; Haack et al. 1999; Fernandez-Carneado et al. 2005). In lipid membranes, the guanidinium group in protein side chain can form a branched moiety by interacting with water and lipid molecules. Mutation of Arg residues (i.e., deletion or replacement) in cationic membrane peptides is usually found to cause significant reduction of their biological activity. Wender et al. showed oligomers of arginine containing dimethylguanidinium head groups have 95 % lower translocation efficiency than the wild type, indicating the importance of this bidentate H-bond donor (Szyk et al. 2006). Thus, charged residues clearly play a crucial role in the biological functions of cationic membrane peptides. However, no high-resolution structure of this distinctive and mechanistically essential complex had been experimentally obtained until recently.

In recent years, SSNMR measurements of our several CPPs and AMPs have identified Arg guanidinium-phosphate and Lys ammonium-phosphate interactions by measuring site-specific distances and dynamics, as summarized in Table 1. The site-specific distance was measured using a SSNMR technique called rotational-echo double-resonance (REDOR) (Gullion and Schaefer 1989; Jaroniec et al. 2001). It uses rotor-synchronized π -pulses to recouple dipolar coupling, which encodes the distance between two nuclear spins. Short site-specific ^{13}C - ^{31}P distance (~ 4 Å) between the distal end carbon (arginine C_{ξ} and lysine C_{ϵ}) of the cationic residues and the phosphate of lipid head-groups provided the most direct molecular-level evidence to validate the existence of H-bonding interaction, since such a short distance is the minimum gap allowed between Arg/Lys and phosphate groups to avoid steric conflict (Su et al. 2009, 2010b).

Figure 1 shows the Arg-/Lys- lipid interactions for penetratin and PG-1, as examples for CPPs and AMPs, respectively (Su et al. 2009, 2010b; Li et al. 2010; Tang et al. 2007). In the ^{13}C - ^{31}P REDOR measurement of the membrane-bound penetratin (Su et al. 2009), Arg10 showed a short C_{ξ} -P distances of 4.3 Å indicating the presence of guanidinium-phosphate complexation (Fig. 1a). Another cationic residue, Lys13 showed a similarly short (4.0 Å) distance between its side chain C_{ϵ} and the lipid ^{31}P (Fig. 1b), indicating Lys-lipid interaction. These ^{13}C - ^{31}P distances, together with geometry parameters from previous studies, lead to the highest resolution structure of Arg-lipid and Lys-lipid interactions so far. In contrast to the short distances observed for cationic residues, a much longer distance of 6.9 Å from the neutral side chain of Ile3 $C_{\gamma/\delta}$ to lipid ^{31}P was observed using double-quantum filtered selective REDOR (Su et al. 2009), indicating that neutral

residues do not contribute to peptide–lipid interactions. The different roles of cationic and neutral residues suggest that the nature of peptide–lipid contact is charge–charge interaction. Interestingly, the guanidinium–phosphate interaction is not only revealed by the short $C\zeta$ –P distance (4.1 Å), but also by the fact that the guanidinium group exhibits higher rigidity than the rest of the Arg side chains in the study of lipid-bound TAT (Su et al. 2010b). The dipolar order parameter (S_{XH}) indicates the motional amplitude of X–H bond and can be measured using the 2D dipolar-chemical-shift correlation (DIPSHIFT) technique. The order parameters of TAT Arg8 decrease from $N\alpha$ ($S_{NH} = 0.20$) to $C\delta$ ($S_{CH} = 0.08$), but then increase to $N\eta$ ($S_{NH} = 0.30$), indicating two relatively rigid ends sandwiching a mobile middle. Thus, the Arg sidechain end experiences stabilizing interactions in the membrane, consistent with the formation of N–H...O–P H-bonds.

Cationic AMPs such as PG-1 and HNP-1 have also shown H-bonding of the guanidinium ion with phosphates (Zhang et al. 2010b; Tang et al. 2008b) (Fig. 1c). ^{13}C – ^{31}P REDOR experiments indicated that PG-1 Arg11 and HNP-1 Arg25 have short $C\zeta$ –P distance of 4.0 Å. The important role of the guanidinium–phosphate interaction was also

revealed by the fact that dimethylation of PG-1 Arg residues to Arg^{mm}, which diminishes the ability of the guanidinium group to act as an H-bond donor, significantly weakened the antimicrobial ability of the peptide.

The fundamental role of guanidinium–phosphate interaction for activities of these cationic membrane peptides was also supported by many molecular dynamics (MD) simulations (Herce and Garcia 2007; Freitas et al. 2005; Yoo and Cui 2008; Vorobyov et al. 2008). For example, in MD simulation of TAT translocation across a DOPC bilayer (Herce and Garcia 2007), Arg–lipid interaction was found to be the driving force for the initial insertion, transient water pore formation and attraction from the phosphates of the distal leaflet. The guanidinium–phosphate complex was also found and proposed to be a crucial interaction for the motion of the gating helix S4 of voltage-sensitive potassium channels (Kv) (Freitas et al. 2005). Our SSNMR study of the Kv S4 helix in lipid bilayers has identified such an interaction at residue Arg8 (Doherty et al. 2010). Overall, the knowledge gained from these experimentally identified intermolecular interactions and from MD simulations, furthered our understanding of protein–lipid interactions and their importance in membrane-active biological systems.

The thermodynamic principles behind membrane insertion of these cationic peptides are important to consider. On one hand, the insertion of cationic residues into a membrane–water interfacial layer appears to be energetically unfavorable due to the Born repulsion (Wimley and White 1996). On the other hand, interactions between the highly charged residues and lipid phosphates and water can contribute to the compensation energy necessary to overcome the insertion energy barrier (Ziegler et al. 2003; Torchilin et al. 2001). The H-bond-stabilized complex is proposed to minimize the polarity of Arg-rich peptides via charge–charge neutralization, which reduces the free-energy barrier from three different aspects: electrostatic interaction between the cationic residues and anionic lipid headgroups, H-bonding within the Arg guanidinium–phosphate complex or the Lys ammonium–phosphate complex, and Arg–water and Lys–water H-bonding. All these interactions have been observed in our SSNMR experiments of CPPs and AMPs (Su et al. 2009, 2010b; Li et al. 2010; Tang et al. 2007).

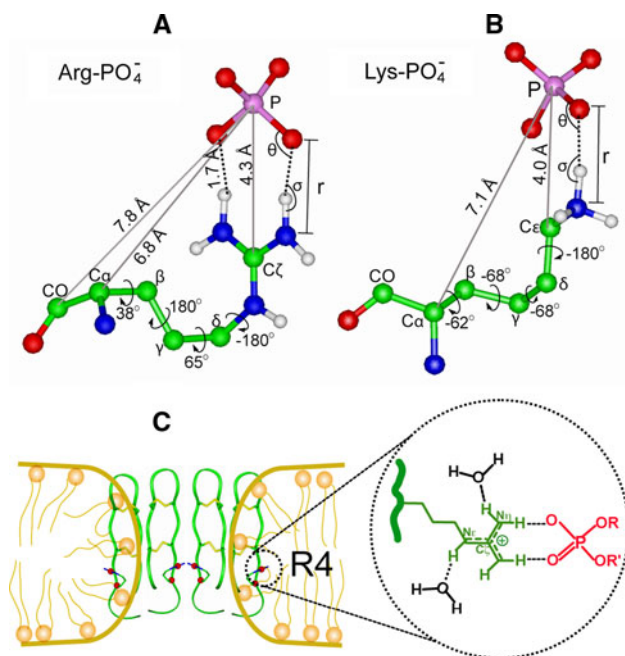


Fig. 1 **a** Arginine–phosphate and **b** lysine–phosphate complexes stabilized by N–H...O–P H-bonds in POPE-POPG-bound CPP penetratin. The structural models were built based on experimentally measured site-specific ^{13}C – ^{31}P distances (solid line) (Su et al. 2011), sidechain dihedral angles from the penultimate rotamer library (Lovell et al. 2000) and N–H...O–P geometric parameters in previous studies (Zhang et al. 1999; de Dios and Oldfield 1994). Geometry parameters of the N–H...O–P H-bonds: $\theta = 120^\circ$, $\sigma = 168^\circ$ and $r = 2.8$ Å. **c** Guanidinium–phosphate and guanidinium–water interactions of POPE-POPG-bound PG-1

Different functional roles of Arg and Lys

Although, both Arg and Lys in CPP sequences play principal roles in cellular uptake, the two residues contribute differently (Wender et al. 2000, 2008; Rothbard et al. 2005; Futaki et al. 2001; Sundlass and Raines 2011; Takechi et al. 2011). Polyarginine (Arg)_n ($n = 7$ or 9), a synthetic CPP with n-consecutive Arg, was reported to enter cells more

efficiently than polylysine (Lys)_n (Mitchell et al. 2000). Wender et al. (2000) showed that the D-arginine oligomer (Arg)₉ exhibited 100-fold higher translocation efficiency than HIV TAT (49–57), and proposed that Arg contributes more than Lys to the cellular uptake. In addition to these differences, TAT and R9 can actively bind to RNA while K9 cannot (Calnan et al. 1991). For AMPs, the Lys-for-Arg mutation also significantly weakens antimicrobial activities. For example, cryptdin-4 (Crp4), a potent bactericidal peptide of the α -defensin family, disrupts the lipid membrane by altering the membrane curvature. A recent study by Schmidt et al. (2012) showed that complete Lys-for-Arg mutation of Crp4 dramatically decreased its antimicrobial potency. However, none of these studies have provided molecular evidence of why Arg and Lys play distinct functional roles.

Although, the NMR-observed ¹³C–³¹P distances indicate strong charge–charge interactions for both Arg10 and Lys13 of penetratin to phosphates of gel-phase lipid membranes, dynamic measurements at physiological temperature showed that Arg10 N η had a much larger order parameter ($S_{\text{NH}} = 0.3$) than the S_{NH} of Lys13 N ϵ which was estimated to be much less than 0.1. This indicates that Lys-phosphate interactions are less stable than Arg-phosphate interactions at physiological temperature. Moreover, Arg10 adopts rigid β -strand conformation at high temperature while Lys13 adopts a relatively mobile β -turn state, which further supports the stronger Arg-lipid interaction. The weaker stability of the Lys-phosphate interaction is reasonable because of the fast rotation of side chain NH₃, which prohibits the formation of stable H-bonding. In comparison, the guanidinium group can form up to four H-bonds with two phosphate groups, creating the so-called guanidinium–phosphate bidentate complex. Therefore, the Lys-phosphate interaction should provide less compensation energy for membrane insertion. Overall, the different stabilities of Arg–lipid and Lys–lipid interactions at physiological temperature explains why lysine substitution of CPPs and AMPs weakens membrane insertion of these peptides. This agrees well with the finding that the inefficient CPP, (Lys)_n, is not able to insert into the membrane, whereas Arg-rich TAT and (Arg)₉ can cross the lipid membrane (Ben-Tal et al. 1996).

Arg-water interaction

The hydration of proteins plays an essential role in the folding, stability, dynamics, and function of proteins, as well as in ligand binding and recognition. So, the investigation of water–protein interactions is necessary for understanding many biological processes. Charged residues such as arginine and lysine are readily exchanged and charge-stabilized with water by forming H-bonds. Thus,

charged residues in membrane proteins are hot spots for interaction with water (Freites et al. 2005). In recent years, several effective SSNMR techniques have been developed to probe protein–water interactions in the lipid membrane environment.

A two-dimensional (2D) ¹H spin diffusion SSNMR method was introduced to semi-quantitatively determine the distances from the mobile lipid chains and water to a rigid membrane-associated protein (Huster et al. 2002). Magnetization initiating from the mobile ¹H magnetization sources such as H₂O and lipid CH₂ groups is allowed to transfer to nearby protein residues. Protein with different depths of insertion will manifest different spin diffusion rates. This technique has been successfully applied to investigate the protein–water interactions in a number of membrane proteins including the influenza A virus M2 protein (Luo and Hong 2010), the chimeric potassium channel KcsA-Kv1.3 (Ader et al. 2008), TAT (Su et al. 2010b) and various AMPs such as tachyplesin-I (TP-1) (Doherty et al. 2006a), PG-1 (Mani et al. 2006) and a charge-reduced PG-1 mutant IB484 (Su et al. 2011). In our TAT study, all polar residues containing labile protons exhibited strong water cross-peaks in the 2D ¹³C-detected ¹H spin diffusion spectrum, indicating stabilization of the charged residues by peptide–water interactions (Su et al. 2010b). Together with the observed guanidinium–phosphate interaction, we proposed that TAT minimizes its hydrophobicity by interacting with lipid phosphate groups and water molecules.

Hetero-nuclear correlation (HETCOR) NMR experiments with ¹H homo-nuclear decoupling have been used to investigate Arg-water interactions in membrane-bound PG-1 (Li et al. 2010; Li and Hong 2011). Arg4 guanidinium–water correlation was clearly detected in the ¹H–¹⁵N Hartman–Hahn (HH)-CP HETCOR spectra, indicating water solvation of this hydrophobically embedded Arg residue (Fig. 1c). This observation of the arginine–water interactions for residues inside the lipid membrane was consistent with MD simulation results (Dorairaj and Allen 2007; Li et al. 2008).

To facilitate the detection of water cross-peaks in ¹³C, ¹⁵N-labeled membrane peptides without perdeuteration in the HETCOR experiments, a ¹³C and/or ¹⁵N dipolar-edited MELODI–HETCOR experiment was introduced to avoid overlap of protein ¹H signals, primarily H α and H^N, with the water signal (Li et al. 2010; Yao et al. 2001). Protein–water interactions can also be manifested from the chemical shift variations of water. Very recently, the histidine–water interaction in the influenza M2 proton channel was experimentally observed from the correlations between the imidazole ring and water at pH ranging from 4.5 to 8.5 in ¹H–¹⁵N HETCOR experiments (Hong et al. 2012a), indicating the presence of strong H-bonding between the histidine side chain and water in the influenza M2 protein.

Cell-penetrating peptides

Since the discovery of cellular uptake of TAT protein in 1988 (Green and Loewenstein 1988; Frankel and Pabo 1988), TAT peptide and many other protein-derived or chemically synthesized CPPs, such as penetratin, transportan and polyarginines, have been widely characterized using biophysical and biochemical techniques, because of great potential for biological research and therapeutic applications. Several CPPs have already entered the clinical phase of animal or human testing (Schwarze et al. 1999; Khafagya et al. 2009; Gratton et al. 2003; Dietz et al. 2008). Despite the extensive biological characterization and applications, the mechanism of translocation still remains unclear because of lack of structure (Christiaens et al. 2002; Persson et al. 2001). By utilizing various solid-state NMR techniques, we have investigated molecular interactions of TAT and penetratin with lipid membranes, which provided valuable information about the relation between their Arg-rich structural properties and membrane translocation (Su et al. 2008a, b, 2009, 2010b; Su and Hong 2011).

Dynamic structure of membrane-bound CPPs: mobility makes opportunity

For the first time, we have determined the conformation of TAT and penetratin in hydrated lipid bilayers using SSNMR (Su et al. 2008a, 2010b). ^{13}C and ^{15}N chemical shifts at physiological temperature indicate penetratin adopts a mobile β -turn rich structure, while TAT shows a random coil conformation. The latter is also supported by

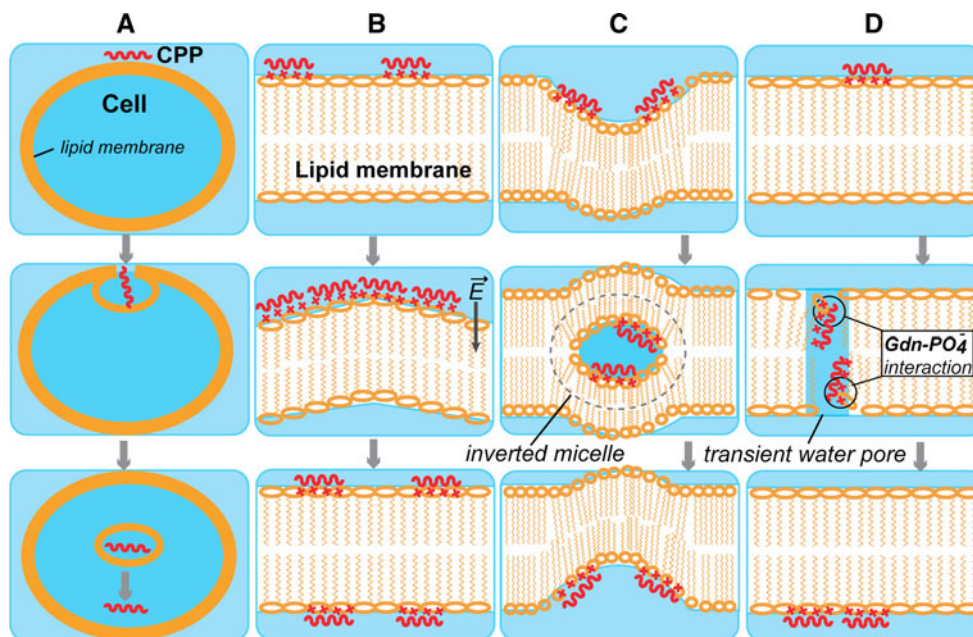
very low backbone order parameters ($S_{\text{CH}} = 0.14\text{--}0.20$), suggesting similarly large mobility as lipids. More recently, the highly dynamic conformations of TAT and penetratin have been detected in live cells by Raman microscopy (Ye et al. 2010) and CD spectroscopy (Guo et al. 2012). Using a novel one-side paramagnetic relaxation enhancement (PRE) method, ^1H spin diffusion and $^{13}\text{C}\text{--}^{31}\text{P}$ REDOR experiments, we found that both peptides insert into the membrane-water interface of the lipid bilayer. Thus, TAT arguably provides the first clear documented case of a membrane-bound random coil peptide (Su et al. 2008b, 2009, 2010b).

CPP translocation mechanism

CPPs are non-invasive and highly efficient intracellular transporters, however, their therapeutic application and development of cell uptake efficiency enhancement are limited by an insufficient understanding of the translocation mechanism. Highly cationic CPPs are extremely unlikely to cross the hydrophobic lipid bilayer by simple passive diffusion without a free-energy penalty. Debate about the various models centers on whether membrane translocation of these peptides proceeds through an inverted-micelle-mediated route, through endocytosis or through direct membrane penetration (Weissig and D'Souz 2012; Madani et al. 2011) (Fig. 2). The endocytosis pathway is proposed based on the fact that CPP uptake in cells was found to adopt an energy-dependent mechanism (Richard et al. 2003) (Fig. 2a). However, cellular uptake is not completely prohibited at low temperatures or in the presence of endocytosis inhibitors (Richard et al. 2003; Letoha

Fig. 2 Schematic models of CPP translocation across the cellular membrane.

- a Endocytosis model.
- b Electroporation model.
- c Inverted-micelle model.
- d Direct penetration model



et al. 2003), suggesting a non-energy consuming route. The electroporation model (Fig. 2b) suggests that at a certain high concentration ($P/L > 1/40$) the cationic peptides induce an electric field through the membrane, which changes membrane curvature and causes electroporation-like membrane permeabilization (Binder and Lindblom 2003). However, our one-side PRE results show that penetratin peptides are located on both leaflets of the bilayer at both low ($P/L = 1:40$) and high ($P/L = 1:20$) peptide concentrations (Su et al. 2008b). The inverted-micelle model invokes the formation of transient vesicles that trap CPPs and escort them across the bilayer (Derossi et al. 1998) (Fig. 2c). However, this model is ruled out by static ^{31}P NMR spectra which preclude any highly mobile isotropic vesicles in lipid membranes in the presence of either penetratin or TAT (Su et al. 2008b, 2010b). Recently, the finding of strong peptide–lipid interactions supports direct translocation of the peptides across the membrane (Fig. 2d) (Herce and Garcia 2007; Szyk et al. 2006; Su et al. 2010b). In addition, many studies also propose that cellular uptake can follow multiple routes depending on experimental conditions, specific CPP sequence and the cargo structure (von Heijne 2006).

Regardless of whether endocytosis is involved or not, CPPs need to penetrate either the plasma or the endosomal membrane to reach the intracellular compartments, therefore, the structural information of CPPs crossing a lipid bilayer from SSNMR studies is valuable for critically evaluating the different models. The direct translocation process stresses the fundamental role of guanidinium–phosphate ion pairs, which have been observed in our TAT and penetratin studies. In the first stage of translocation, CPPs bind to the lipid membrane surface. The high affinity of CPP for the membrane surface results from electrostatic interaction, and was confirmed in many thermodynamic and MD studies (Jing et al. 2012; Mitchell et al. 2000; Torchilin et al. 2001; Ziegler et al. 2003). Subsequently, the H-bonding interactions of Arg guanidinium and lipid phosphate facilitate the insertion of the surface-bound peptides. The experimentally observed complex makes CPPs more membrane-soluble by minimizing the exposure of the charged groups to the hydrophobic environment. H-bonding of the guanidinium group with lipid phosphate and water also provides sufficient compensation energy to overcome the Born repulsion from the membrane-water interface. The membrane-water interfacial insertion of TAT and penetratin has been identified in our studies. The movement of CPPs inside the lipid membrane may be facilitated by the transient water pore formation, as proposed by Herce and Garcia in their MD simulation of TAT translocation (Herce and Garcia 2007) and indicated by the strong guanidinium–water interaction, we observed (Su et al. 2010b). Meanwhile, peptide concentration difference

at various membrane depths is caused by the accumulation of peptide near the outer membrane surface. In addition, CPPs also experience charge–charge interaction from the distal phosphate layer. Possibly driven by these two factors, the phosphate- or water-neutralized peptides could move in the intracellular direction and finally accomplish the translocation. Overall, the guanidinium–phosphate interactions stabilize the CPP peptides in lipids and facilitate the insertion, while the plastic conformation and high mobility further promote the translocation.

Antimicrobial peptides

To date, hundreds of AMPs have been isolated from a variety of organisms ranging from microbes to plant and animals. Their biological and chemical properties have been extensively characterized in the past 20 years. In general, they have a high content of cationic residues, adopt amphipathic conformation and carry out antimicrobial actions by permeabilizing the microbial cell membrane. However, how these highly charged peptides insert into the lipid membrane seems puzzling. This question can hardly be answered without knowledge of the structure and dynamics of AMPs in the lipid membrane. Here, we will use PG-1 and its mutants as examples to summarize SSNMR findings of the structure, topology and dynamics of the family of β -hairpin AMPs, and most importantly to show how this structural information can be correlated to antimicrobial potency and selectivity.

Structure and topology of AMPs in lipid bilayers

Over the past few years, we used SSNMR to study many AMPs, including retrocyclin-2, tachyplesin-I, HNP-1, PG-1 and its mutants (Tang and Hong 2009; Doherty et al. 2006a, b; Buffy et al. 2004; Hong and Su 2011; Su et al. 2010a). In contrast to unstructured CPPs, AMPs normally adopt well-defined secondary structures to conduct their antimicrobial activity in lipid membranes. As a representative cationic cysteine-rich AMP, PG-1 adopts a β -hairpin conformation due to its two intramolecular disulfide bonds (Fahrner et al. 1996). The knowledge of structural behaviors of AMPs in hydrated lipid membrane, such as insertion depth and aggregation of peptides, is more valuable. For example, PG-1 in DPC micelles exists as anti-parallel NCCN dimers (Roumestand et al. 1998), while our intermolecular distance measurements show that PG-1 peptides pack as parallel NCCN dimers in POPC and POPE-POPG lipid bilayers (Mani et al. 2006). Different lipid compositions of the membrane bilayer cause distinct PG-1 oligomerization states and insertion depths. Our SSNMR data show PG-1 peptides form a toroidal β -barrel pore in a

transmembrane fashion in bacteria-mimetic anionic lipids (i.e. POPE/POPG), but stay on the membrane surface as β -sheet aggregates in eukaryotic cell-mimetic cholesterol-rich membranes (i.e. POPC/cholesterol) (Mani et al. 2006).

Antimicrobial mechanisms

AMPs selectively kill bacteria mainly by disrupting cytoplasmic membrane integrity, and sometimes by altering the membrane potential. The first step towards membrane disruption is the high affinity of the cationic peptides on the anionic lipid membrane surface due to electrostatic interactions. Many studies have shown that the charge density affects the membrane-binding ability and in turn the antimicrobial activity (Balali-Mood et al. 2003; Dathe et al. 2001). In addition to charge, structural properties such as conformation, hydrophobicity and amphipathicity of the peptide also contribute to the antimicrobial potency and mechanism (Yeaman and Yount 2003).

In recent years, several models have been suggested to rationalize the mechanism of action of AMPs. These include the barrel-stave model, the toroidal pore model, the carpet model, and the in-plane diffusion or partial-insertion model (Fig. 3a–d) (Bechinger 1999; Brogden 2005; Epanand and Vogel 1999). These four models are distinguished by the peptide location in the membrane, peptide oligomeric structure, dynamics, and the membrane disorder. The barrel-stave model (Fig. 3a) involves a few peptides forming a barrel to span the lipid bilayer, and was proposed based on the studies of alamethicin using neutron scattering and single-channel conductance techniques (Baumann and Mueller 1974; Bechinger 1999). The toroidal pore model addresses amphipathic AMP interaction with lipids through

a torus-shaped transmembrane channel (Fig. 3b). It was first proposed to explain the antimicrobial activity of magainin (Ludtke et al. 1996; Matsuzaki et al. 1994). Both the barrel-stave and the toroidal pore models posit a transmembrane pore, but the latter involves significant orientational changes of lipid head groups from their normal lamellar bilayer orientations, such that the lipids merge the two leaflets of the membrane to form a pore. ^{13}C -detected ^1H spin diffusion experiments showed that POPE-POPG-bound PG-1 is in close contact (2 Å) with the lipid chain methyl groups, which represent the membrane center strongly indicating that PG-1 adopts a transmembrane orientation. Together with the finding that some lipid headgroup are embedded in the hydrophobic region of the membrane (Tang et al. 2007), we propose that PG-1 forms a ‘toroidal pore’ to kill microbial cells (Mani et al. 2006; Tang et al. 2007). In the carpet model (Fig. 3c), AMPs aggregate on the membrane surface at a certain high peptide concentration causing membrane thinning and eventual micellization (Shai and Oren 2001). In the in-plane diffusion or partial-insertion model (Bechinger 1999), surface-bound AMPs do not aggregate but induce local curvature of the lipid membrane (Fig. 3d). The fast diffusion of peptide triggers transient pores, which can cause sufficient disruption to lead to loss of cell viability. AMPs like TP-1, which is too short to span the bilayer to induce transmembrane pores and does not aggregate, adopt such an in-plane diffusion mechanism (Doherty et al. 2006a).

Mechanism of double antimicrobial selectivity

AMPs show double selectivity, i.e., different antimicrobial potency in bacterial and mammalian membranes and

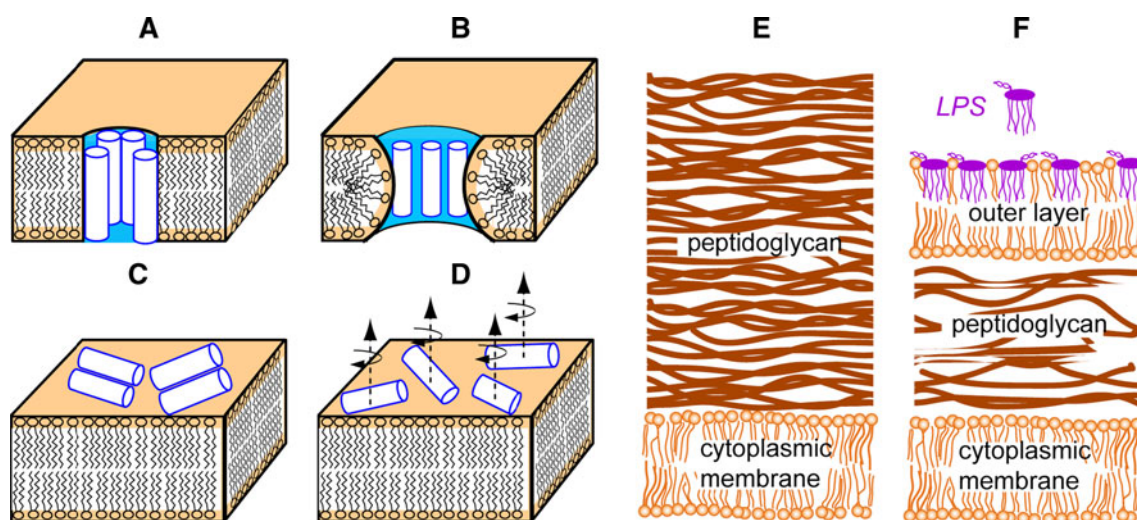


Fig. 3 Membrane disruption models (a–d) of AMPs and Gram-positive (e) and Gram-negative (f) bacterial membranes. **a** Barrel-stave transmembrane model. **b** Toroidal pore model. **c** Carpet model.

d In-plane diffusion or partial-insertion model. AMP examples for each model are given in the text

antimicrobial selectivity against gram-positive versus gram-negative bacteria. Different membrane compositions of bacterial and mammalian membranes help to make the peptides active towards microbial invaders and less harmful to the host cells. Bacterial membranes are rich in anionic phospholipids, while mammalian cell membranes consist of zwitterionic phospholipids (Rouser et al. 1968). The recognition of the former is realized by the preferred binding of positively charged peptides to negatively charged lipids. In addition, the higher anionic lipid content of bacterial membranes can also facilitate the insertion of cationic AMPs. In addition to anionic lipid content, cholesterol, a main component in mammalian cell membranes, is absent in bacterial cells. It is known that cholesterol can rigidify the lipid membrane, protecting the host cell from AMP insertion.

These hypotheses have all been proven to be true by many others' work as well as our SSNMR study of PG-1 in various lipid membranes. PG-1 is active against various bacteria and fungi and the MICs ranging 0.3–3 μM , but it is almost inactive in mammalian cells. SSNMR ^{31}P line-shapes of lipid membranes containing PG-1 indicate that the peptide preferentially disrupts negatively charged membranes (Yamaguchi et al. 2002; Buffy et al. 2003). The membrane-selective disruption was suggested in studies of the insertion depth of PG-1; the bacteria-mimetic anionic POPE-POPG membrane allows peptide insertion, while the eukaryote-MIMIC POPC-cholesterol membrane prevents PG-1 insertion (Mani et al. 2006; Glukhov et al. 2005).

In addition to the selectivity between bacterial and mammalian cells, many AMPs show different antimicrobial potency in gram-positive versus gram-negative bacteria. The two types of bacteria are distinguished by the structural differences of their cell walls (Fig. 3e, f). Both kinds of bacteria have a cytoplasmic membrane layer composed of phospholipids, where most AMPs conduct their antimicrobial activity (Tang and Hong 2009). The difference is that the gram-negative bacterial membrane has an extra outer membrane, which is composed of lipopolysaccharide (LPS) in the outer leaflet and phospholipids (mostly PE) in the inner leaflet (Sperandeo et al. 2009). PG-1, active in both kinds of bacteria, and IB484, a charge-reduced PG-1 mutant that is only potent in gram-positive bacteria, have provided ideal model systems to understand the antimicrobial selectivity between the two types of bacteria (Chen et al. 2000). Our SSNMR results of the topological structure and interaction of the two peptides showed that both peptides insert into the center of the gram-positive-bacteria mimetic POPE-POPG membrane and cause lipid headgroup reorientation, consistent with the toroidal pores. However, PG-1 fully inserts into the gram-negative-bacteria mimetic ReLPS-DEPE membranes,

whereas IB484 does not, indicating that the antimicrobial potency is modulated by insertion of the peptide in the outer membrane (Su et al. 2011). The bulky size of LPS makes the outer layer less permeable than the cytoplasmic membrane of gram-positive bacteria. Due to the largely reduced charge intensity, IB484 has relatively weak electrostatic attraction to the LPS layer, which prevents its further insertion into the cytoplasmic membrane of the gram-negative bacteria. Our comparative study of PG-1 and IB484 indicates that the charge density of AMPs is a crucial structural property determining their antimicrobial selectivity against gram-positive and gram-negative bacteria.

CPPs and AMPs: how different are they?

The comparison between CPPs and AMPs suggests that the conformation and dynamics are the structural factors distinguishing the two categories of membrane peptides. Most AMPs adopt rigid amphipathic secondary structures, either α -helical or in β -sheet (White and Wimley 1999; Abbassi et al. 2008; Mangoni et al. 2000; Ulmschneider and Ulmschneider 2008; Thundimadathil et al. 2006; Steiner et al. 1981; Zasloff 1987). However, the two studied CPPs, TAT and penetratin, show a turn-rich conformation and random coil structure in lipid bilayers, respectively, suggesting that the absence of intra- or intermolecular H-bonded conformation and high molecular mobility may be the hallmarks of CPPs differentiates them from AMPs.

Due to the similar Arg-rich structural motif, CPPs and AMPs have strong Arg–lipid and Arg–water interactions, which stabilize these hydrophobic peptides by membrane neutralization and water solvation, and thus facilitate the insertion. However, CPPs insert into the cellular membrane in a non-invasive manner, while AMPs function by disrupting the lipid membrane. For CPPs, a small guanidinium N–H order parameter ($S_{\text{NH}} = 0.30$) was obtained for both penetratin Arg10 and TAT Arg8, indicating that Arg–lipid H-bonding is relatively weak and will not retard the mobility of CPPs in the lipid membrane. The dynamic structure may serve to prevent these Arg-rich CPPs from permanently residing in and damaging the lipid membrane, thus facilitating peptide across the lipid bilayer. For AMPs, as represented by PG-1, their backbone is largely immobilized due to intra- or intermolecular H-bonds and oligomerization. The high backbone rigidity of aggregated PG-1 in POPE-POPG membranes is indicated by SSNMR results of large dipolar order parameters ($S_{\text{XH}} = 0.70\text{--}1.05$) (Tang et al. 2008a). Thus, the stable transmembrane insertion and lipid interaction together with the formation of oligomerized structures such as β -barrels cause membrane disorder and pore formation, resulting in membrane disruption.

Conclusion and perspectives

We have carried out many comprehensive solid-state NMR studies to investigate the conformation, dynamics and depth of insertion of representative CPPs and AMPs in the lipid bilayer. The mechanistically crucial guanidinium–phosphate and guanidinium–water interactions have been experimentally identified. These atomic-level structural results have contributed significantly to uncover the underlying mechanisms of CPP translocation and AMP antimicrobial activity, and gain insights into developing alternative compounds with more potent activities for pharmaceutical applications. While the lipid-bound studies provided plausible information on the equilibrium structures of these membrane peptides to elucidate the insertion route of CPPs and AMPs, knowledge of *in situ* translocation structures will be important (Ye et al. 2010). Thus, further understanding of the membrane insertion mechanism requires key intermediate structures and interactions, which are likely invisible in the equilibrium state, to be identified *in vivo*. One strategy is to trap transient stages in membrane insertion by rapid sample freezing on the 10–20 μ s time scale (Hu et al. 2010). The low-intensity difficulty in the structural characterization due to the distribution of conformational intermediates may be overcome using DNP-enhanced SSNMR (Robert 2010).

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