



Membrane Permeabilization Mechanisms

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Abstract

Many antimicrobial peptides are considered to kill microbes by permeabilizing cell membranes. This chapter summarizes the driving force of peptide binding to membranes; various mechanisms of lipid bilayer permeabilization including the barrel-stave, toroidal pore, and carpet models; and modes of permeabilization of bacterial and mammalian membranes.

Keywords

Membrane permeabilization · Membrane binding · Membrane curvature · Barrel-stave model · Toroidal pore model · Carpet model

bility in minutes (Matsuzaki et al. 1997a). Rapid killing is an important self-defense action of AMPs. Observations that enantiomeric peptides composed of all D-amino acids are equipotent to the parent L-peptides indicate that proteins requiring chiral recognition such as receptors and enzymes are not involved in the membrane permeabilization process (Bessalle et al. 1990; Wade et al. 1990). Furthermore, magainins induce the leakage of water-soluble dyes entrapped in artificial lipid vesicles (Matsuzaki et al. 1989, 1991a), suggesting that the lipid matrix of membranes is a target of the peptides. Many AMPs such as tachyplesin I (Matsuzaki et al. 1991b) and LL-37 (Lee et al. 2011) also permeabilize membranes.

2.1 Introduction

Membrane permeabilization as a mechanism for bacterial killing has already been suggested in a paper on the discovery of magainins from the African clawed frog *Xenopus laevis*, an archetypical antimicrobial peptide (AMP) isolated from a vertebrate for the first time (Zasloff 1987). As shown in Fig. 2.1, the addition of magainin 2 to *E. coli* cells induced an efflux of intracellular K⁺ ions and concomitantly a decrease in cell via-

2.2 Membrane Binding

The first step in the membrane permeabilization process is the binding of peptides to membranes. AMPs are generally polycationic and amphipathic. In many cases, linear peptides take unordered structures in an aqueous solution, whereas they are conformed to be amphipathic secondary structures, typically α -helices or β -strands, upon membrane binding (Matsuzaki et al. 1989, 1991a). Such amphipathic structures fit the membrane–water interface. Even conformationally restricted cyclic peptides also change their structures to accommodate themselves to membrane environments (Imura et al. 2007).

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The main driving forces for membrane binding are electrostatic attraction and hydrophobic interaction (Matsuzaki et al. 1995a, 2009). Positively charged peptides preferentially interact with negatively charged membranes. Bacterial membranes are rich in acidic phospholipids (phosphatidylglycerol and cardiolipin). Furthermore, cell walls containing lipopolysaccharides (LPS) and peptidoglycans are also negatively charged. In contrast, mammalian cell membranes are less negatively charged. Acidic phospholipids, such as phosphati-

dylserine, are essentially sequestered on the cytoplasmic face of the membrane, although cancer cells show an increased exposure of phosphatidylserine (Utsugi et al. 1991). However, gangliosides containing sialic acid residues have some negative charges on the cell surface, which are a target of AMPs (Miyazaki et al. 2012). Interestingly, acidic phospholipids and gangliosides interact differently with AMPs (Fig. 2.2). Cationic peptides specifically bind to gangliosides containing anionic sialic acid residues so that the charge is neutralized. The binding is described by the Langmuir-type equation. Fluorescent resonance transfer experiments clearly revealed that AMPs preferentially interacted with monosialoganglioside GM1 compared with phosphatidylcholine in a GM1/phosphatidylcholine mixed bilayer mimicking mammalian cell membranes. In contrast, peptides equally interact with anionic phosphatidylglycerol and zwitterionic phosphatidylcholine in a phosphatidylglycerol/phosphatidylcholine mixed membrane, a model for bacterial cell membranes (Miyazaki et al. 2012). The interaction is theoretically explained by a combination of the Gouy-Chapman theory (electrostatic concentration immediately above the membrane surface) and a partition equilibrium (Wenk and Seelig 1998; Wieprecht et al. 1999).

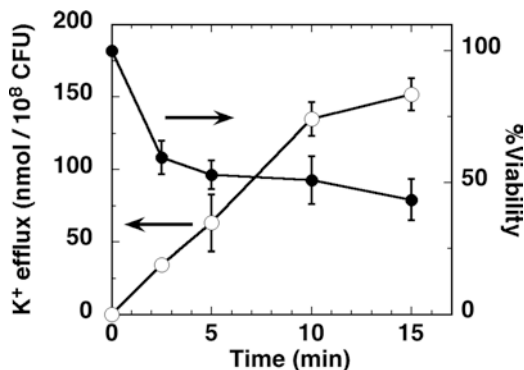


Fig. 2.1 Correlation between membrane permeabilization and bacterial death. Magainin 2 (50 nmol) was added to *E. coli* cells (5×10^8 CFU/mL). K⁺ efflux (open circles, left axis) and the percent cell viability (closed circles, right axis) are plotted as a function of time

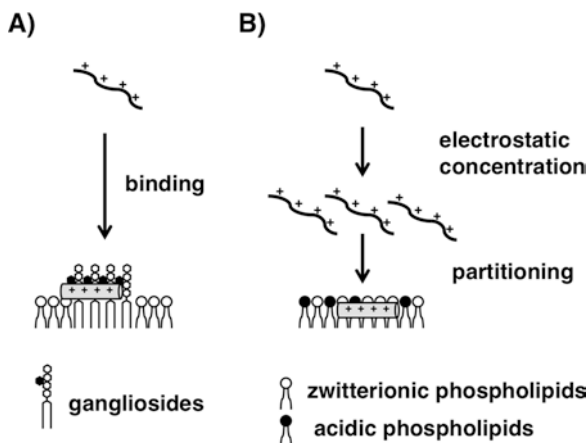


Fig. 2.2 Different binding modes of AMPs to (a) mammalian and (b) bacterial model membranes. (a) Cationic AMPs specifically bind to gangliosides containing anionic sialic acid residues. The binding is described by the Langmuir-type equation. (b) In contrast, peptides are

electrostatically concentrated immediately above the membrane surface according to the Gouy-Chapman theory and then partitioned into the membrane. There is no specific interaction between AMPs and acidic phospholipids

2.3 Permeabilization of Model Membranes

Membrane-bound AMPs change membrane structures and organizations leading to membrane permeabilization. Using model membranes such as liposomes, several mechanisms so far proposed for this can be roughly classified into two categories, i.e., membrane curvature modulation and phase separation. The recently proposed latter mechanism includes clustering of acidic

lipids by cationic AMPs and is described in Chap. 5. The “barrel-stave channel,” “toroidal pore,” and original “carpet” mechanisms are categorized as the former mechanism (Fig. 2.3). Refer to Chap. 3 and a review by Huang (2006) for theoretical treatments.

Amphipathic secondary structures on the surface of membranes can modulate membrane curvature. The peptides expand the interfacial region, making a void in the hydrocarbon region of the membrane. Consequently, membrane-

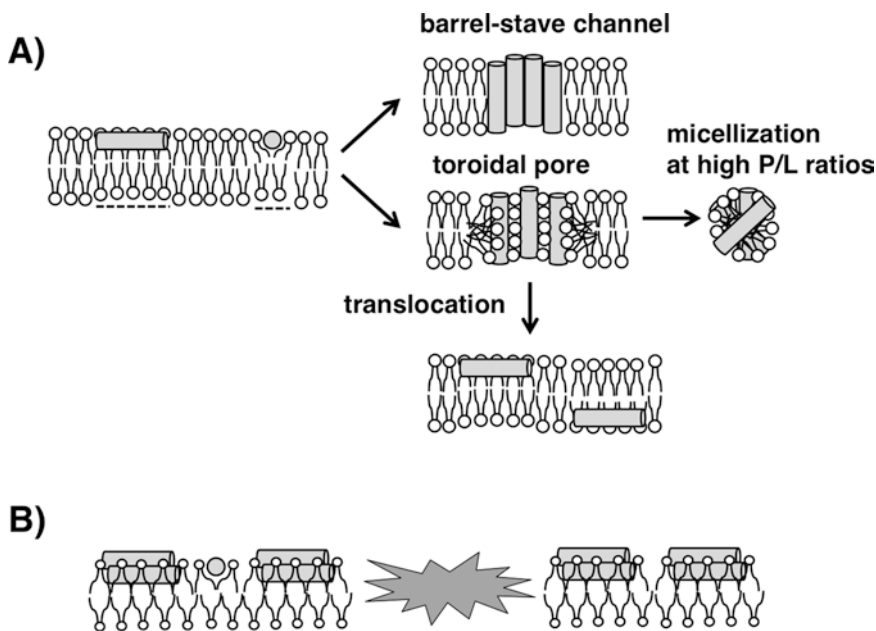


Fig. 2.3 Major mechanisms of membrane permeabilization induced by AMPs. (a) AMPs form amphipathic secondary structures (α -helix in this case) on the surface of membranes. The peptides expand the interfacial region making a void in the hydrocarbon region of the membrane. Consequently, membrane-thinning and positive curvature (concave) strain are induced (broken line). At threshold peptide-to-lipid ratios, typically below $\sim 1:100$, either the “barrel-stave channel” or the “toroidal pore” is formed. The former is solely composed of peptides, making a water-filled channel. The ionic current is discrete and the conductance depends on the number of peptides involved in a single channel. A typical example of this class of peptide is the peptaibol alamethicin. However, most AMPs form a toroidal pore, in which both the polar faces of the amphiphilic structures and the polar headgroups of lipids constitute the pore wall. This unique structure allows not only the passage of ions and small molecules through the pore but also the rapid flip–flop of lipids along the pore

wall. The pore is not stable (the lifetime is typically ms), and upon its disintegration a fraction of peptide molecules translocates across bilayers. At much higher peptide-to-lipid ratios, membranes are solubilized into micelles. (b) When membranes have a negative-curvature (convex) tendency, it counteracts the positive curvature induced by the peptides, stabilizing the peptide–lipid system and allowing accumulation of large amounts of peptides. Eventually (e.g., peptide-to-lipid ratios above 1:10), membrane disruption occurs. This mechanism corresponds to the original “carpet model.” It should be noted that the modified carpet model also includes toroidal pore formation, although its scientific validity needs careful consideration. Similar phenomena can happen in zero-curvature bilayers with peptides having a large hydrophobic surface capable of expanding the hydrocarbon core of the membrane. Thus, the mechanism of membrane permeabilization is not unique to peptides but also depends on the physicochemical properties of membranes

thinning and positive curvature (concave) strain are induced (Fig. 2.3a left). At threshold peptide-to-lipid ratios, typically below $\sim 1:100$, the surface-lying peptides are cooperatively inserted into the membrane, forming a water-filled pore (Fig. 2.3a right). Peptaibols, peptides containing **Aib** (aminoisobutyric acid) residues with the C-terminal alcohol, have been known to form a barrel-stave channel (Sansom 1991). These peptides are electrostatically almost neutral. A typical example is the antibiotic alamethicin produced by the fungus *Trichoderma viride*. The channel is solely composed of helical peptides. The ionic current is discrete and the conductance depends on the number of peptides involved in a single channel. Magainins were also considered to form this type of channel at the time of its discovery. However, we noticed that this was not the case and proposed the toroidal pore model in 1996 (Matsuzaki et al. 1996a).

First, in the case of the barrel-stave channel, the size of the pore depends on the peptide-to-lipid molar ratio. However, as shown in Fig. 2.4, the small I^- ion and the medium-sized calcein dye leaked out of liposomes in the same range of peptide-to-lipid ratio, and larger dye-labeled dextran molecules were retained even at higher peptide-to-lipid ratios, suggesting that a pore of defined size (diameter 2–3 nm) was formed.

Second, the leakage kinetics were unique. The percent leakage value appeared to reach a plateau instead of complete leakage, indicating that the

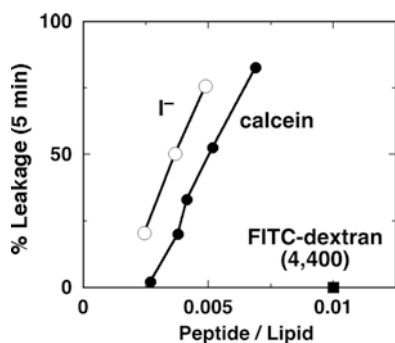


Fig. 2.4 Estimation of the pore size formed by magainin 2. The percent leakage values are plotted as a function of the peptide-to-lipid ratio for I^- ions (open circles), calcein (MW 623, closed circles), and FITC-dextran (MW 4400, closed squares)

number of pores decreased with increasing time (Fig. 2.5). We hypothesized that pores were unstable and a fraction of peptide molecules was translocated into the inner leaflet. Thus, the peptide density in the outer leaflet decreased, decelerating pore formation. Indeed, the translocation was coupled to the leakage. Kinetic analysis suggested that a pore is composed of ~ 5 magainin molecules, which appeared to be too small to allow the passage of calcein (Matsuzaki et al. 1995b). Thus, we hypothesized that lipid molecules were also involved in the pore structure and examined the flip-flop of lipids. The flop was again coupled to leakage and translocation (Fig. 2.5). The flip rate was identical to the flop rate and did not depend on the type of lipid, suggesting that all lipid molecules in the membrane were randomized (Matsuzaki et al. 1996a). Based on these observations, we proposed the toroidal pore model, in which both the polar faces of the amphiphilic helices and the polar headgroups of lipids constitute the pore wall. This unique structure allows not only the passage of ions and small molecules through the pore but also the rapid flip-flop of lipids along the pore wall. Both types of events seem to contribute to bactericidal activity. The flop of phosphatidylserine was also observed in mammalian cells (Imura et al. 2008). The structure of the toroidal pore was confirmed by neutron scattering experiments by Huang's group (Ludtke et al. 1996).

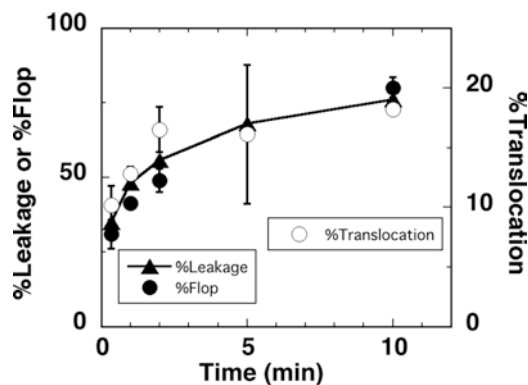


Fig. 2.5 Coupling between leakage, peptide translocation, and lipid flop. The percent leakage of calcein (solid line), percent peptide translocation (open circles), and percent flop of NBD-phosphatidylethanolamine (closed circles) are plotted as a function of time

Not only magainins but also other AMPs and membrane-acting peptides form toroidal pores, including helical PGLa (Matsuzaki et al. 1998a), mastoparan X (Matsuzaki et al. 1996b), melittin (Yang et al. 2001), buforin 2 (Kobayashi et al. 2004), and cyclic β -sheet tachyplesin I (Imura et al. 1768). The pore size depends on the type of peptide. The two substitutions F5Y and F16W for magainin 2 enlarged the pore size to 4–7 nm (Hara et al. 2001). The bacteriocin lacticin Q also forms a large toroidal pore of similar size (Yoneyama et al. 2009).

The mode of dye leakage is classified into an all-or-none or graded mode. In the former, some vesicles are empty, whereas the rest are intact. In the latter, all vesicles partially lose their contents. Although these modes are sometimes considered to originate from different leakage mechanisms, earlier theoretical studies showed that the pore lifetime τ determines the mode of leakage (Schwarz and Arbuza 1995; Schwarz and Robert 1992). The all-or-none and the graded mode correspond to $\tau \gg \tau_0$ (the time necessary for a $1/e$ reduction in the intravesicular dye concentration) and $\tau \ll \tau_0$, respectively.

The pore lifetime is governed by electrostatics within the pore and is therefore modulated by both peptide charge and lipid composition because the toroidal pore is composed of peptides and lipids. An increase in peptide positive charge destabilizes the pore because of enhanced electrostatic repulsion between closely spaced peptides, facilitating peptide translocation (Matsuzaki et al. 1997b). Note that translocation occurs only upon the disintegration of the pore. For example, an increase in the positive charge of magainin from +4 to +6 reduced the τ value from $9\tau_0$ to $0.1\tau_0$. Buforin 2 with +6 charges is translocated across liposomal (Kobayashi et al. 2000) and bacterial (Park et al. 1998) membranes without permeabilizing them. Similarly, an increase in acidic lipid content stabilizes the pore by reducing electrostatic repulsion between peptides in a pore (Kobayashi et al. 2004). Dye leakage and lipid flip–flop are observed in this case.

Another well-known mechanism of membrane permeabilization is the original “carpet model,” in which peptides cover the membrane surface like a

carpet and disrupt the bilayer organization (Shai 1995) (Fig. 2.3b). This model was proposed following studies on the interaction between cecropins and dermaseptins with phosphatidylserine-containing bilayers. We found that even magainins do not form toroidal pores, but accumulate on the membrane surface and eventually disrupt it at peptide-to-lipid ratios above 1:10 in membranes containing phosphatidylserine (Fig. 2.6), phosphatidic acid, or cardiolipin (Matsuzaki et al. 1998b). These lipids form hexagonal-II phases under charge-neutralizing conditions and thus tend to induce negative curvature strain on the membrane, counteracting the positive curvature strain imposed by magainins and allowing accumulation of huge amounts of peptides on the membrane. Thus, the mechanism of membrane permeabilization is not unique to peptides, but also depends on the physicochemical properties of membranes. It should be noted that the current modified carpet model also includes toroidal pore formation (Oren and Shai 1998), although its scientific validity needs careful consideration. Recently, the detergent-like model (Bechinger and Lohner 2006) and the interfacial activity model (Wimley 2010) have been proposed for the mechanism of AMPs. These more comprehensive models are similar to the curvature modulation mechanism described above in that the incorporation of amphipathic structures in lipid bilayers modifies the bilayer organization.

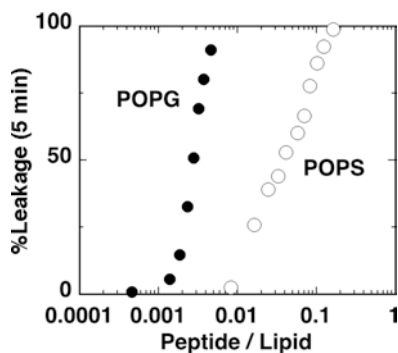


Fig. 2.6 Dependence of magainin 2 leakage activity on lipid species. The percent leakage values are plotted as a function of the peptide-to-lipid ratios in a logarithmic scale for palmitoyloleoylphosphatidylglycerol (POPG, closed circles) and palmitoyloleoylphosphatidylserine (POPS, open circles)

2.4 Permeabilization of Bacterial Membranes

AMPs have been proposed to disrupt and permeabilize the outer membrane of Gram-negative bacteria by the “self-promoted pathway” (Hancock and Chapple 1999). Cationic peptides compete with Mg^{2+} ions that bridge between adjacent phosphates of LPS. Magainin forms a helix upon binding to the lipid A moiety of LPS (Matsuzaki et al. 1999) and induces blebs on the outer membrane, while the presence of Mg^{2+} ions inhibits its antimicrobial activity (Matsuzaki et al. 1997a). The permeabilization of the outer membrane can be detected by the permeation of impermeable substances such as nonionic detergents (Matsuzaki et al. 1997a) and 1-*N*-phenyl-naphthylamine (NPN) (Zhang et al. 1999). Recently, a method based on the leakage of green fluorescent protein (GFP) expressed in the periplasmic space was developed (Sochacki et al. 2011). Note that the diameter of the protein (4.5 nm) is larger than that of the magainin toroidal pore (2–3 nm).

The permeabilization of cytoplasmic membranes can be monitored by several techniques. The efflux of intracellular K^+ ions is detected by a K^+ -selective electrode (Matsuzaki et al. 1997a). Potential sensitive dyes, such as diSC₃5, detect the dissipation of transmembrane potential (Patzrykat et al. 2002). The hydrolysis of *o*-nitrophenyl- β -D-galactoside (ONPG) by cytoplasmic β -galactosidase is also often utilized for *E. coli* ML-35 (*lacI*⁻, *lacY*⁻, *lacZ*⁺). More convenient methods include observation of the cell entry of water-soluble dyes such as calcein (Imura et al. 2008) and Sytox Green (Sochacki et al. 2011) by fluorescent microscopy.

Which mechanism works better for the interaction of AMPs with bacterial membranes is a matter of attention (Wimley 2010; Roversi et al. 2014). Generally, the amount of peptide bound to bacterial cells at its minimum inhibitory or bactericidal concentration (MIC or MBC) is considered. However, it should be noted that MIC or MBC is the concentration needed to inhibit the growth of or to kill the most resistant population of bacteria and is thus too strong for the other

more susceptible populations. The experimental conditions in Fig. 2.1 may be appropriate for discussion because about 50% of bacteria are killed. Assuming that (1) peptide molecules are intact and completely bound to both leaflets of the outer and inner membranes, (2) the binding to other bacterial components is negligible, and (3) the number of lipids per cell is 4×10^7 (Roversi et al. 2014), the peptide-to-lipid ratio in the inner membrane is ~ 1 . To examine the direct interaction of magainin 2 with inner membranes, we used *E. coli* spheroplasts lacking outer membranes (Matsuzaki et al. 1997a). The peptide lysed spheroplasts in a peptide-to-lipid ratio range of 0.1–1, in accordance with the rough estimate above. The lipid composition of the spheroplasts was 68% phosphatidylethanolamine/24% phosphatidylglycerol/8% cardiolipin. Asymmetry of lipid distribution in the inner membrane has not yet been observed (Furse and Scott 2016). Nevertheless, even if the inner leaflet is composed of phosphatidylethanolamine alone, the outer leaflet is composed of $\sim 50\%$ phosphatidylglycerol and $\sim 50\%$ lipids with a negative-curvature tendency (cardiolipin and phosphatidylethanolamine). Thus, the original carpet mechanism is more likely, although further studies are needed to confirm this. Transmission electron microscopy revealed that LL-37 caused local perturbations and breaks along *P. aeruginosa* cell membranes (Andersson et al. 2004). However, the mechanism of membrane permeabilization may depend on the type of AMPs, as well as bacterial strains. Magainin appears to form a toroidal pore against the Gram-positive *B. megaterium* because the peptide forms membrane lesions of definite size (~ 2.8 nm, < 6.6 nm) (Imura et al. 2008).

2.5 Permeabilization of Mammalian Cell Membranes

Relatively little is known about how AMPs permeabilize and are translocated across mammalian cell membranes. Our earlier work suggested that prototypical membrane-permeabilizing

magainin 2 and intracellular-targeting buforin 2 interact with human cells differently (Takeshima et al. 2003). Dye-labeled magainin was translocated across HeLa cells via both energy-dependent and energy-independent mechanisms, and the internalization was accompanied by cytotoxicity. In contrast, dye-labeled buforin penetrates cells in an energy-independent fashion and exerts little toxicity. Zanetti's group reported that the Pro-rich peptide Bac7 (1–35) is taken up by murine and human cells through a nontoxic energy- and temperature-dependent process (Tomasinsig et al. 2006). Details on so-called cell-penetrating peptide (CPP) are described in Chap. 7.

2.6 Conclusion

Thirty years of extensive studies, both experimental and theoretical, have revealed several basic mechanisms for the permeabilization of lipid bilayers by AMPs, as described above. It depends on both peptide sequences and lipid compositions. However, the molecular details are difficult to understand because initial membrane permeabilization is often dynamic and transient in nature, whereas spectroscopic methods usually detect equilibrium states after the permeabilization ceases. In contrast to model membrane studies, little is known on the permeabilization mechanisms of bacterial and mammalian cells, which remain subjects of further study.

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