Elucidation of Complex Dynamic Intermolecular Interactions in Membranes

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Biomembranes composed of various proteins and lipids play important roles in cellular functions, such as signal transduction and substance transport. In addition, some bioactive peptides and pathogenic proteins target membrane proteins and lipids to exert their effects. Therefore, an understanding of dynamic and complex intermolecular interactions among these membrane constituents is needed to elucidate their mechanisms. This review summarizes the major research carried out in the author’s laboratory on how lipids and their inhomogeneous distributions regulate the structures and functions of antimicrobial peptides and Alzheimer’s amyloid β-protein. Also, how to detect transmembrane helix–helix and membrane protein–protein interactions and how they are modulated by lipids are discussed.

Key words antimicrobial peptide; amyloid β-protein; transmembrane helix; membrane protein; peptide–lipid interaction

1. Introduction

Biomembranes play important roles in cellular functions, such as signal transduction and substance transport. The basic architecture of biomembranes is the lipid bilayer, in which various functional proteins are incorporated. Human erythrocyte membranes, one of the best-studied biomembranes and a model of mammalian plasma membranes, contain several hundred lipid species, the distribution of which is longitudinally asymmetric (Fig. 1). The outer leaflet contains mainly zwitterionic lipids, such as phosphatidylcholine (PC) and sphingomyelin (SM) with negatively charged sphingoglycolipids including gangliosides as minor components, whereas phosphatidylethanolamine (PE) and acidic phospholipids, such as phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA), are localized in the inner leaflet.1)

Recently, the lateral distribution of lipids was also proposed to be non-uniform (Fig. 1). Sphingolipids (SM and sphingoglycolipids) and cholesterol form so-called ‘lipid raft’ microdomains, in which proteins involved in signal transduction are concentrated.2) Not only such diversity in lipid species and their inhomogeneous distributions but also physicochemical properties of lipid bilayers, e.g., membrane thickness and fluidity, also regulate the structure and function of membrane proteins.3)

This review summarizes the major research carried out in the author’s laboratory on how the asymmetry and lateral heterogeneity of lipid distribution regulate the structures and functions of antimicrobial peptides and Alzheimer’s amyloid β-protein, respectively. Furthermore, methods to detect transmembrane helix–helix and membrane protein–protein interactions and how they are affected by membrane thickness and cholesterol are discussed.

2. Antimicrobial Peptides

Antimicrobial peptides (AMPs) are cationic and amphiphilic peptides typically composed of 15–50 amino acid residues produced by living organisms including humans as part of innate immunity and also promising candidates for antibacterial drugs.4–6) The cationicity is important for their selective interaction with bacterial cell surfaces because bacterial surfaces are more negatively charged than mammalian cell surfaces (Fig. 2). The bacterial cell membranes are rich in anionic phospholipids, mainly phosphatidylglycerol (PG) and cardiolipin (dimerized PG). Anionic peptidoglycan layers cover bacterial cell membranes. In addition, Gram-negative bacteria have outer membranes coated with negatively charged lipopolysaccharides (LPS). The majority of AMPs exert antimicrobial activity by permeabilizing membranes.7) This general mode of action endows them with the important properties: broad antimicrobial spectra and difficulty of emergence of resistant bacteria. For more details, refer to review articles by the author.8–12)

We have investigated interactions of several AMPs with lipid bilayers as a model of cell membranes as well as bacteria to elucidate their molecular mechanisms of action (Table 1). The major driving forces of membrane binding are electrostatic and hydrophobic interactions. AMPs, the hydrophobicity of which is generally low, exhibit relatively weak interaction with zwitterionic lipids and therefore normal mammalian cells. It should be noted that the hydrophobicity of amino
acid residues depends on their positions along the peptide sequence with terminal positions contributing less to the overall hydrophobicity.\(^{13}\) AMPs selectively bind to negatively charged bilayers and bacterial cell membranes by electrostatic interaction\(^{14–18}\) (Fig. 3). Interestingly, however, fluorescent resonance energy transfer (FRET) experiments between the Trp residue incorporated in magainin 2 and pyrene-labeled lipids revealed that there is no specific interaction between the peptides and anionic lipids. Rather, AMPs are electrostatically concentrated above the membranes and then partitioned into the membranes.\(^{19}\) The membrane-bound peptides form amphiphilic secondary structures, typically \(\alpha\)-helices, and are located at the water–membrane interface.\(^{15,20}\) In contrast, the \(\alpha\)-helical magainin 2 peptide is specifically bound to the sugar group region of monosialoganglioside GM1, which is overexpressed on the surface of tumorigenic HeLa cells.\(^{19}\) Such specific interaction is also observed between tachyplesin I having a cyclic \(\beta\)-sheet structure and LPS.\(^{21}\)

Bound peptides, which are often partially oligomerized,\(^{17,22}\) expand the headgroup region of bilayers, inducing membrane thinning\(^{23}\) and positive curvature strain,\(^{24}\) leading to the formation of the unique toroidal pore, in which not only the

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**Biography**

Katsumi Matsuzaki was born in 1959 in Osaka. He received his bachelor’s and master’s degrees in Pharmaceutical Science at Kyoto University in 1982 and 1984, respectively, under the supervision of Professor Masayuki Nakagaki. After he worked for Takeda Chemical Industries Co., he returned to his school in 1987 as an Assistant Professor (Professor Koichiro Miyajima). He received his Ph.D. from Kyoto University in 1992, and then worked for 10 months as a Visiting Scientist at the Biocenter of the University of Basel, Switzerland (Professor Joachim Seelig). He was appointed as an Associate Professor of the Graduate School of Pharmaceutical Sciences, Kyoto University in 1997. He moved to the newly-established Graduate School of Biostudies, Kyoto University (Professor Yasunori Kozutsumi) in 1999. He has been a Professor of the Graduate School of Pharmaceutical Sciences, Kyoto University since 2003. His research interests include biophysical studies on interplays among peptides, proteins, and lipids in membranes. He received The Japanese Peptide Society Award for Young Scientists in 1996, The Pharmaceutical Society of Japan Award for Young Scientists in 1997, Erwin von Bälz Prize in 2011, and The Pharmaceutical Society of Japan Award in 2021.
hydrophilic faces of the amphiphilic secondary structures but also the headgroups of lipids constitute the pore lumen\(^{25-27}\) (Fig. 4). The structure markedly contrasts with the already known 'barrel-stave' ion channel formed by peptibols, such as alamethicin\(^{28}\) and hypelcin A\(^{29,30}\) in the presence of transmembrane voltage. Approximately 5 helices are involved in a pore formed by magainin 2 and its diameter is 2–3 nm,\(^{12,31,32}\) although the pore size is sensitive to subtle amino acid substitutions.\(^{33}\) This structure makes otherwise independent outer and inner leaflets a continuum, allowing a rapid scrambling of lipids.\(^{25,32}\) The pore formation rate increases with the decreasing polar angle of the amphiphilic helix.\(^{34}\) The lifetime of the pore is short (approx. ms) and decreases with an increasing number of positive charges.\(^{17,35}\) Upon its disintegration, some peptides are translocated into the inner leaflet.\(^{25,27,36}\) Buforin 2 (Table 1) is known to cross membranes without their permeabilization and interact with intracellular DNA/RNA.\(^{37}\) Its mechanism of translocation is the same as that of magainin 2: The extremely short lifetime of the pore prevents membrane permeabilization and enables effective translocation.\(^{38}\) The toroidal pore is also formed by the wasp venom mastoparan X\(^{39}\) and bee venom melittin.\(^{40,41}\) It should be noted that in lipid bilayers with a negative curvature strain, e.g., PS, the peptide-induced positive curvature strain is counteracted by surrounding lipids, resulting in membrane disruption due to the accumulation of large numbers of peptides.\(^{24}\)

A combination of two AMPs occasionally exhibits synergistic activity. Magainin 2 and PGLa, both from the same origin (Table 1), form a 1:1 complex, in which the two helices align in parallel, and exhibit much higher antimicrobial activity but also greater cytotoxicity than the individual components alone.\(^{17,42-44}\) Another example is a mixture of magainin 2 and tachyplesin I, which exhibits bacteria-selective synergism, although the underlying mechanism is remains unclear.\(^{45}\)

For the development of anti-infective drugs, improvement of the therapeutic index is crucial. The attachment of polyethylene glycol (MW 5000) at the N-terminus of tachyplesin I can almost nullify its cytotoxicity at the expense of significant reduction in antimicrobial activity.\(^{27}\) The drawback can be partially overcome by the augmentation of positive charges, as exemplified in the case of magainin 2.\(^{46}\)

Anticancer agents are another application of AMPs because the surfaces of cancer cells are more negatively charged than normal cells\(^{47,48}\) (Fig. 3). AMPs that are activated at a weakly acidic pH would show augmented selectivity for cancer cells because the pH in tumor tissue (5.6–6.8\(^{39}\)) or 6.2–6.9\(^{49}\)) is

### Table 1. Amino Acid Sequences of AMPs Investigated

| Peptide     | Origin                        | Sequence                                      |
|-------------|-------------------------------|-----------------------------------------------|
| α-Helical   |                               |                                               |
| Magainin 1  | *Xenopus laevis*              | GIGKF LHSAG KFGKA FVGEI MKS                   |
| Magainin 2  |                               | GIGKF LHSAK KFGKA FVGEI MNS                   |
| PGLa        |                               | GMASK AGAIA GKIAG VALKA L-amide               |
| Buforin 2   | *Bufo bufo gargarizans*       | TRSSR AGLQF PVGRV HRLLR K                     |
| Cyclic-β-sheet |                               |                                               |
| Tachyplesin I | *Tachypleus tridentata*      | KWCFR VCY R                                 |

Fig. 3. Molecular Basis of Cell Selectivity of AMPs

AMPs preferentially interact with bacterial and cancer cells over normal cells by virtue of electrostatic interaction. The mode of action is, however, different between bacterial and cancer cells. See the text for details.
lower than that in normal tissue (approx. 7.4). For this purpose, the charge-reversal peptide HE (GIHHWLHSAH EFGEH FVHHI MNS-amide) was designed using magainin 2 as a template. Its charge reverses from $-1.5$ at pH 7.4 to $+6$ at pH 5.5. The peptide exerted moderate toxicity against human renal adenocarcinoma ACHN cells at pH 6.0 (EC$_{50}$ approx. 100 µM), but not at least up to 100 µM at pH 7.4, and was nontoxic against human normal glomerular mesangial cells even at this low pH. To enhance tumoricidal activity, we introduced 2,3-diaminopropionic acid (Dap) residues with a pK$_a$ value of 6.3 instead of His. The best peptide with 8 Dap residues (GIXXW LHSAX XFGXX FVXXI ZNS-amide, $X = $ Dap, $Y = $ norleucine) exhibited EC$_{50}$ values of approx. 5 and 60 µM against the multidrug-resistant human pancreas carcinoma cell line PANC-1 at pH 6.0 and 7.4, respectively. Furthermore, the introduction of Dap also reduced cytotoxicity against normal HEK293 cells and glomerular mesangial cells at pH 7.4 (EC$_{50}$ > 100 µM).

3. Alzheimer’s Amyloid β-Protein

Alzheimer’s disease (AD) is a major form of dementia, and amyloid β-protein (Aβ), most commonly composed of 40 or 42 amino acid residues, plays a central role in its pathogenesis. Alzheimer’s disease (AD) is a major form of dementia, and amyloid β-protein (Aβ), most commonly composed of 40 or 42 amino acid residues, plays a central role in its pathogenesis. Self-assembled forms of Aβ rich in β-sheet structures (oligomers and fibrils) impair neuronal cells. Aβ mainly originates from the neuronal cell surface amyloid precursor protein, which is sequentially cleaved by β- and γ-secretases in recycling endosomes, and is most likely released into the synaptic terminal. Thus, Aβ has a better chance to interact with neuronal membranes. Accumulating evidence has suggested that the binding of Aβ to membranes plays an pivotal role in the aggregation of Aβ.

Yanagisawa et al. identified a specific form of Aβ bound to the monosialoganglioside GM1, a sphingoglycolipid abundant in neuronal membranes (Fig. 5(b)), in brains exhibiting the early pathological changes associated with AD, and also suggested that the GM1-bound form of Aβ may serve as a seed for the formation of Aβ aggregates. Inspired by this discovery, we have systematically investigated Aβ–membrane interaction using lipid bilayers as well as neuronal cells. The membrane-mediated amyloidogenesis mechanism also explains the reason why aged rodents rarely develop the characteristic lesions of AD. Rodent Aβ forms much less toxic fibrils on neuronal cells than its human counterpart. Our major findings are illustrated in Fig. 6, and also refer to reviews by the author for more details. Aβ does not interact with membranes without GM1 or with uniformly distributed GM1, corresponding to the healthy condition. An increase in
the cholesterol level triggers the formation of GM1 clusters, which are recognized by Aβ.67–72) The protein initially forms an α-helical form, which is converted to a β-sheet rich oligomer composed of 10–20 Aβ molecules with an increase in the protein density in the membrane.73,74) Interestingly, this oligomer is nontoxic, in marked contrast to toxic oligomers formed in aqueous solution.74) A further increase in the protein concentration leads to the formation of toxic amyloid fibrils,75–77) which bind to a complex of toll-like receptors 4, 6, and CD36, inducing the nuclear translocation of nuclear factor-kappaB (NFκB), sequentially activating the NLRP3 inflammasome and caspases-8, -9, and -2, finally resulting in apoptosis.78)

The harmful fibril has a unique structure, which is a flat, tape-like structure composed of a single β-sheet layer formed by mixed in-register parallel and 2-residue-shifted antiparallel structures.79) The latter configuration is stabilized by multiple salt bridges and hydrophobic/π–π interactions80) (Fig. 5(c)). In low polarity environments in membranes, electrostatic interactions are much stronger than in aqueous solution, strengthening the salt bridge interaction in the 2-residue-shifted antiparallel arrangement, compensating for unfavorable repulsive forces between adjacent charged amino acid residues in the in-register parallel structure. Similar amyloid fibrils are also formed in a less polar 1, 4-dioxane/water mixture.81) Trace amounts of pyroglytamated Aβ-(3–42) on neuronal membranes at physiological nanomolar concentrations,82) Fibrl formation in aqueous solution and membranes can be inhibited by nanogels,83) a helical Aβ analog,84) and small compounds.85)

4. Transmembrane Helices

The folding and stability of α-helical membrane proteins, such as G-protein-coupled receptors (GPCR), are governed by interactions between their constituent transmembrane helices.86,87) An attractive approach to measure transmembrane helix–helix interactions is the use of model peptides forming transmembrane helices. First, we designed the completely hydrophobic, ‘inert’ peptide (LALAAA A)3 without any of the specific sidechain interactions and showed that they form a stable transmembrane helix, which can be inserted into and dissociated from lipid bilayers.88,89) The insertion topology can be controlled by helix macrodipole–transmembrane potential interaction. Systematic investigation of the thermodynamics of transmembrane helix–helix interactions was carried out using the modified peptide (ALAALA)3 with a symmetric sequence. FRET measurements between dye-labeled peptides revealed that the helices form antiparallel dimers with a crossing angle of approx. 0° by electrostatic (enthalpic) helix macrodipole interactions. In thinner membranes, the helix termini having partial charges causing the macrodipole are exposed to the aqueous phase with a high polarity, diminishing the helix–helix interaction, whereas in thicker membranes they are buried in a less polar environment, leading to a stronger helix dimerization90,91) (Fig. 7(a)). Changes in hydrophobic thickness of dimonounsaturated PC bilayers from C14 to C22 caused an alteration in the ΔG value from −10 to −20 kJ mol−1 at 308 K. The presence of PE having a smaller headgroup than PC92) and cholesterol93) enhanced the antiparallel dimer formation, which is enthalpically driven. Interestingly, in the presence of cholesterol, the dimer is X-shaped with a crossing angle of 56°, because cholesterol with the small polar group (-OH) and large hydrophobic sterol ring imposes a high lateral pressure in the central part of bilayers.

A GXXXG motif is known to mediate interhelical packings in helical membrane proteins.94) To examine the effects of the introduction of the motif on the self-association of transmem-
brane helices, single-pair FRET experiments were devised using a pair of topologically controlled helices incorporated in a liposome. The host peptide \((\text{ALAALA})_3\) did not form a parallel dimer because of repulsive macrodipole interaction. In contrast, the \(\text{AALALAA-AGLALGA-AALALAA}\) peptide with the motif in the middle as the guest self-associated with a small crossing angle of 10° in PC bilayers (Fig. 7(b)). The incorporation of cholesterol destroyed this dimer structure because it is destabilized by the high lateral pressure in the hydrophobic region of the bilayer. Thus, helical assembly is controlled by not only amino acid sequences but also lipid compositions.

## 5. Membrane Proteins

Membrane proteins, such as receptors and ion channels, often function as homo- or hetero-oligomers. Existing approaches have a number of problems in precisely determining the oligomeric states of membrane proteins on the plasma membranes of living cells. Destructive methods such as immuneprecipitation can detect artificial aggregation of proteins in the presence of detergents. As nondisruptive approaches, FRET and bioluminescence resonance energy transfer have been widely employed to monitor protein–protein interactions in living cells. Most studies use the genetic fusion of fluorescent or luminescent proteins to label the target proteins. However, the considerable size of the label may perturb protein functions. Furthermore, it is difficult to control the donor/acceptor expression ratios, which are critical to quantitatively analyze the results. In addition, signals from proteins that are not sorted to the plasma membrane hamper accurate analysis. To overcome these problems, we developed a novel labeling technique named the ‘coiled–coil labeling method’ (Fig. 8). It utilizes the tight heterodimer formation between the negatively charged \(\text{E3 tag} (\text{EIAALEK})_3\) and the positively charged \(\text{K probe} (\text{KIAALKE})_x\) \((x = 3 \text{ or } 4)\). The E3 tag is genetically fused to the target protein, which is labeled with donor- and acceptor-attached K probes.
can be avoided by incorporating phosphoserine residues in the probe.\textsuperscript{103} Selective covalent labeling of amine groups of cell surface proteins is also possible, guided by the coiled–coil assembly.\textsuperscript{104}

Using this approach combined with a spectral imaging technique (in-cell FRET), we have elucidated the oligomeric states of various membrane proteins in living cells. Regarding membrane receptors, the metabotropic glutamate receptor forms a dimer,\textsuperscript{105} whereas class-A GPCRs including $\beta_2$-adrenergic receptor,\textsuperscript{106} chemokine-CXCR4, dopamine-D2, and prostaglandin-EP1 receptors are monomeric.\textsuperscript{106} Epidermal growth factor receptor internalization, which accompanies a reduction in endosomal pH, can be detected as an increase in the tetramethylrhodamine probe.\textsuperscript{107} Selective covalent labeling of amine groups of cell membranes and cholesterol removal is needed to induce its dimerization.\textsuperscript{108}

The coiled–coil method can also be used to monitor receptor internalization. $\beta_2$-Adrenergic receptor was doubly labeled with a mixture of pH-sensitive fluorescein and pH-insensitive tetramethylrhodamine.\textsuperscript{109} Agonist-induced receptor internalization, which accompanies a reduction in endosomal pH, can be detected as an increase in the tetramethylrhodamine-to-fluorescein fluorescence intensity ratio. Furthermore, the screening of agonists and antagonists is possible by utilizing the translocation of the receptor from cell-surface to intracellular regions and the acidification in endosomes.\textsuperscript{110}

6. Conclusion
Elucidation of interactions among peptides, proteins, and lipids in membranes is intractable because they are complex and time-dependent. We have clarified various physicochemical phenomena in the membrane milieu by the combined use of spectroscopic, thermodynamic, kinetic, chemical, and molecular biological approaches. Molecular mechanisms proposed by liposomal studies were confirmed by cellular experiments as much as possible. Especially, the involvement of peptide/protein–lipid interactions in innate immunity (AMPs) and the pathogenesis of AD was revealed in detail. Live-cell FRET experiments using the coiled–coil technique suggested the importance of nondisruptive, less perturbing methods to investigate protein–protein interaction in cell membranes. The author sincerely hopes that our achievements contribute to the further understanding of membrane-staged biological phenomena and the development of potent anti-infective agents and disease-modifying drugs for AD.

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Conflict of Interest  The author declares no conflict of interest.

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