Role of membrane lipids in the mechanism of bacterial species selective toxicity by two α/β-antimicrobial peptides

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Abstract

We have previously shown that two synthetic antimicrobial peptides with alternating α- and β-amino acid residues, designated simply as α/β-peptide I and α/β-peptide II, had toxicity toward bacteria and affected the morphology of bacterial membranes in a manner that correlated with their effects on liposomes with lipid composition similar to those of the bacteria. In the present study we account for the weak effects of α/β-peptide I on liposomes or bacteria whose membranes are enriched in phosphatidylethanolamine (PE) and why such membranes are particularly susceptible to damage by α/β-peptide II. The α/β-peptide II has marked effects on unilamellar vesicles enriched in PE causing vesicle aggregation and loss of their internal aqueous contents. The molecular basis of these effects is the ability of α/β-peptide II to induce phase segregation of anionic and zwitterionic lipids as shown by fluorescence and differential scanning calorimetry. This phase separation could result in the formation of defects through which polar materials could pass across the membrane as well as form a PE-rich membrane domain that would not be a stable bilayer. α/β-Peptide II is more effective in this regard because, unlike α/β-peptide I, it has a string of two or three adjacent cationic residues that can interact with anionic lipids. Although α/β-peptide I can destroy membrane barriers by converting lamellar to non-lamellar structures, it does so only weakly with unilamellar vesicles or with bacteria because it is not as efficient in the aggregation of these membranes leading to the bilayer–bilayer contacts required for this phase conversion. This study provides further understanding of why α/β-peptide II is more toxic to micro-organisms with a high PE content in their membrane as well as for the lack of toxicity of α/β-peptide I with these cells, emphasizing the potential importance of the lipid composition of the cell surface in determining selective toxicity of anti-microbial agents.

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1. Introduction

It is well known that the chemical and physical nature of the cell surface differs widely among different cell types. Much attention has been given to the greater extent of exposure of anionic charge on the surface of bacterial cells compared with mammalian cells. This negative charge is the basis of the microbial specificity of many antimicrobial peptides, which are cationic and therefore bind preferentially to the anionic surface of bacteria. There are also several studies demonstrating lipid head group specificity, apart from charge, in the interactions of antimicrobial peptides with model membranes, see for example [1,2]. However, this description is not complete since it is known that many antimicrobial peptides show selective toxicity for certain bacterial species. Furthermore, the species most sensitive to a particular agent differs for different antimicrobial peptides. Hence, there must be other factors determining toxicity in addition to the charge on the cell surface. One consideration is that not all of the antimicrobial peptides act by...
permeabilizing the cell membrane [3]. Nevertheless, the membrane must play some role in targeting of peptides to different cell types, irrespective of the mechanism of cell killing. Gram positive and Gram negative bacteria differ fundamentally in the morphology of their surfaces (Fig. 1). Gram negative bacteria have an outer membrane that is rich in lipopolysaccharides in addition to the cytoplasmic membrane. These bacteria also have a peptidoglycan layer between the two membranes. In contrast, peptidoglycan makes up the cell wall of Gram positive bacteria and contains teichoic and lipoteichoic acids. The peptidoglycan layer of Gram positive bacteria is much thicker, 20–80 nm, than in Gram negative bacteria where it is found to be about ten times smaller [4]. Neither the outer membrane of Gram negative bacteria nor the cell wall of Gram positive bacteria are generally considered to be a major barrier to the penetration of peptides into the cell. The major exceptions to this are peptides that bind to components of the cell wall or outer membrane, which is not the case for the peptides used in the present study [5].

We have been studying a pair of oligomers that contains sequentially alternating α- and β-amino acid residues (“α/β-peptides”), I and II (Fig. 2). The peptides are shown both as a linear sequence. There is evidence that this class of peptides forms i, i+4 C═O–H–N backbone hydrogen bonds, which occur in alternating 14- and 15-membered rings (“14/15-helix”) [6]. Although α/β-peptides I and II have identical chemical composition and identical charge, they have very different potencies with different cell types (Table 1). α/β-Peptide II is much more toxic to E. coli than is α/β–peptide I, while α/β–peptide I is more lytic toward human erythrocytes. The phospholipid composition of cell membranes can differ dramatically, even among bacteria (Table 2). Human erythrocytes exhibit a large difference in the lipid composition of the two monolayers of the cell membrane bilayer, with the cytoplasmic surface having most of the phosphatidylethanolamine (PE) and anionic lipid, while the extracellular monolayer has essentially all of the sphingomyelin (SM) and is devoid of anionic lipid. In the case of E. coli (Gram negative), the major lipid in both monolayers is PE, while for B. subtilis (Gram positive) the major lipids are the anionic phosphatidylglycerol (PG) and cardiolipin (CL). There are thus major differences in the phospholipid composition of the exposed membrane surface among these three cell types (Gram negative bacteria, Gram positive bacteria, and erythrocytes), and even larger differences between the two types of bacteria themselves.

There is a phenomenological correlation between the toxicity of α/β-peptides I and II toward these three cell types and the rate of aqueous contents leakage induced by these peptides in liposomes with a lipid composition corresponding to that of a particular cell type [5]. Thus α/β–peptide I is more hemolytic (Table 1) and induces greater leakage in liposomes devoid of anionic lipid, while α/β–peptide II is toxic to E. coli and causes

![Fig. 1. Schematic representation of the membrane organization of Gram positive and Gram negative bacteria.](http://www.stanford.edu/~amatin/MatinLabHomePage/Student%20Presentation/Zomora/Zamora%20handout.pdf)
reverse-phase HPLC mobility: α-peptide II. This physicochemical difference is manifested in activities of these molecules toward cells. We show that these differences can explain variations in the present report we investigate further the differences in the approach of adjacent membranes, both with model vesicles as well as between the inner and outer membranes of E. coli. Both should form a helix containing α/β- peptides I and II. Both should form a helix containing α/β- peptides I and II. In this conformation, α/β-peptide I should display one large hydrophobic patch, but the hydrophobic side chains of sequence isomer α/β-peptide II should be clustered into smaller patches.

We have previously shown that α/β-peptide II causes closer approach of adjacent membranes, both with model vesicles as well as between the inner and outer membranes of E. coli [5]. In the present report we investigate further the differences in the interactions of these two peptides with PE-rich membranes, and we show that these differences can explain variations in the activities of these molecules toward cells.

2. Materials and methods

2.1. Materials

Phospholipids, including the fluorescently labeled lipids, were purchased from Avanti Polar Lipids (Alabaster, AL). The synthesis and purification of α/β-Peptide I and II have been described [6].

2.2. Preparation of LUV

Lipid films were made by dissolving appropriate amounts of lipid in a mixture of chloroform/methanol, 2/1 (v/v), followed by solvent evaporation under nitrogen to deposit the lipid as a film on the wall of a test tube. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap, for 2–3 h. Dried films were kept under Argon gas at −30 °C if not used immediately. Films were hydrated with 10 mM HEPES, 0.14 M NaCl, 1 mM EDTA (HEPES buffer) and vortexed extensively at room temperature. The lipid suspension was then subjected to five cycles of freezing and thawing and the homogeneous lipid suspensions were then further processed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA) in a high pressure barrel extruder (Lipex Biomembranes, Vancouver, BC), at room temperature to extrude LUVs. The size of the LUVs were monitored with quasi-elastic light scattering, as previously described [7], and found to have a diameter of 90±10 Å. The vesicles were kept on ice and used within a few hours of preparation. Lipid phosphorus was determined by the method of Ames [8].

2.3. Liposome aggregation measured by turbidity changes

Absorbance at 436 nm was measured in a Cary 50 Bio UV-visible spectrophotometer, as a function of time, after peptide addition to a suspension of 50 μM LUVs composed of DOPE:DOPG (2:1) in HEPES buffer, pH 7.4. The rate of change of turbidity was measured at 30 °C, with constant stirring. Zero absorbance was set with LUVs, prior to addition of peptide.

2.4. Liposome aggregation measured by confocal fluorescence microscopy

Films composed of DOPE:DOPG (2:1) containing 1 mol% of Rh-PE were hydrated with HEPES buffer pH 7.4 and LUVs were made by extrusion. To a 50 μM suspension of LUVs, 10 μM α/β-peptide I or II in HEPES buffer were added. A drop of this mixture was placed in a glass slide, overlaid with a coverslip and then visualized by confocal fluorescence microscopy using a Zeiss Axiosvert laser scanning confocal inverted microscope (LSM-510). Fluorescence from Rh-PE was monitored using a 505–530 nm bandpass filter in the excitation path. Images were analyzed with the program Zeiss LSM 5 Image Browser version 2.80.112.3.

2.5. Fluorescence assay for the lateral segregation of lipids

LUVs were prepared in HEPES buffer from lipid films containing 1 mol% N-Rh-PE and 1 mol% C6-NBD-PG, C6-NBD-PC or C6-NBD-PE. Fluorescence spectra were measured as a function of time with the liposomes contained in cuvettes in 2 mM HEPES buffer, pH 7.4, 25 °C. The excitation wavelength was 465 nm and a 500 nm cut off filter was used with a 4-nm bandpass in excitation and emission. 50 μM LUVs were titrated with incremental amounts of peptides in HEPES buffer solution. The spectra were measured between 500 nm and 650 nm after each addition of peptide.

### Table 1

<table>
<thead>
<tr>
<th>Antibacterial and hemolytic activities of α/β-peptides</th>
<th>(\alpha/\beta)-Peptide I</th>
<th>(\alpha/\beta)-Peptides II</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>E. coli[^a^]</td>
<td>B. subtilis[^b^]</td>
<td>1.6</td>
</tr>
<tr>
<td>(\alpha/\beta)-Peptide I</td>
<td>&gt;100</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>(\alpha/\beta)-Peptides II</td>
<td>6.3</td>
<td>6.3</td>
<td>50</td>
</tr>
</tbody>
</table>

[^a^]: From Schmitt et al. [6].
[^b^]: Minimum Inhibitory Concentration (μg/mL).
[^c^]: Maximum concentration without hemolysis (μg/mL).

### Table 2

<table>
<thead>
<tr>
<th>Major phospholipid components</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
<th>PC</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli inner membrane [29]</td>
<td>80</td>
<td>15</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. subtilis cell membrane [30]</td>
<td>12</td>
<td>70</td>
<td>4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Human erythrocyte [31]</td>
<td>29</td>
<td>(13)</td>
<td>–</td>
<td>30</td>
<td>27</td>
</tr>
</tbody>
</table>

[^a^]: Total anionic lipid = PS + PI + PA.
2.6. Differential Scanning Calorimetry (DSC)

Lipid films were prepared as for LUVs and were then hydrated with 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN₃, pH 7.40. Measurements were made using a Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation, American Fork, UT). The scan rate was 0.75 °C/min and there was a delay of 5 min between sequential scans in a series to allow for thermal equilibration. The features of the design of this instrument have been described [9]. DSC curves were analyzed by using the fitting program, DA-2, provided by Microcal Inc. (Northampton, MA) and plotted with Prism, version 4.01.

3. Results

3.1. Role of α/β-peptides in promoting the aggregation of LUVs

We previously observed that α/β-peptide I could induce the formation of interlamellar contacts between bilayers enriched in PE, resulting in the formation of a morphology similar to a bicontinuous cubic phase but without long range order [5]. Such a morphology is referred to as a sponge phase [10]. Formation of this phase either from LUVs or from cell membranes requires membrane–membrane contact. We therefore monitored the ability of these peptides to induce the aggregation of LUVs. We have assessed the potency of α/β-peptides I and II to promote vesicle aggregation by measuring the time dependent increase in turbidity at 436 nm after addition of one of these oligomers. Aggregation of LUVs composed of DOPE:DOPG (2:1) occurs very rapidly and is concentration dependent in both cases. The rate and extent of aggregation are much greater for α/β-peptide II than for α/β-peptide I, and significant LUV aggregation occurs at much lower concentrations of α/β-peptide II than of α/β-peptide I (Fig. 3A and B). Additional studies indicated that the nature of the lipid is crucial for induced aggregation. A smaller degree of aggregation was detected with DOPC:DOPG (2:1) LUVs, and not much aggregation was seen with DOPC LUVs, upon addition of up to 10 μM of either of the α/β-peptides. Note that vesicle aggregation is required for leakage caused by the formation of inverted phases, but is not required for other mechanisms of peptide-induced leakage, such as pore formation or the carpet mechanism. Therefore, α/β-peptide I must induce hemolysis by a mechanism not involving bilayer–bilayer contact or inverted phase formation. These results are thus consistent with the finding that α/β-peptide I is hemolytic.

3.2. Redistribution of phospholipids in the plane of the membrane measured by FRET

Peptide-induced segregation of lipids into domains can destabilize the membrane by introducing phase boundary

Fig. 3. (A) Aggregation of LUVs as a function of peptide concentration and different times studied by absorbance at 436 nm, at 30 °C, in DOPE:DOPG (2:1) LUVs. Top panel, α/β-peptide I. Bottom panel, α/β-peptide II. The decrease in aggregation at 300 s with α/β-peptide II reflects the clumping of some aggregated material. (B) Aggregation of LUVs of DOPC:DOPE (2:1) containing 1 mol% of Rh-PE at 150 s after addition of α/β-peptides I or II, as seen by confocal fluorescence microscopy. As LUVs aggregate, more particles become visible as small dots. Top panel, α/β-peptide I. Bottom panel, α/β-peptides II. Calibration bar is 2 μm.
defects between lipid domains [11–16]. In addition, in lipid mixtures with a high content of PE, the formation of a PE-rich domain will cause bilayer destabilization because of the high negative curvature tendency of PE lipids. Fluorescence resonance energy transfer (FRET) from C6-NBD-PG, C6-NBD-PE or C6-NBD-PC to N-Rh-PE was used to assess the ability of α/β-peptides I and II to cause segregation of PG vs. PC or PE into domains. The N-Rh-PE was present in all samples together with one of the two NBD-labeled lipids. Decrease in FRET is detected as a decrease in rhodamine fluorescence. FRET will decrease if the donor or acceptor is sequestered into a segregated domain. The representative anionic lipid probe is C6-NBD-PG and that for the zwitterionic component is C6-NBD-PC or C6-NBD-PE. In all cases the NBD group is on the acyl chain. The Rh-PE is the common FRET acceptor for the NBD lipid probes and it has the fluorophore on the lipid headgroup, making this lipid anionic. If the Rh-PE is sequestered by the cationic peptide it will decrease FRET equally with both the anionic and zwitterionic lipid probe. If the cationic peptides preferentially sequester C6-NBD-PG, FRET with this lipid will be decreased more than with C6-NBD-PC or C6-NBD-PE. \(I_o\) is the intensity of fluorescence emission from Rhodamine in the absence of peptide and \(I\) the intensity in the presence of the concentration of peptide given in the abscissa. The ratio \(I/I_o\) decreases as the peptide is added (Fig. 4). The differences in FRET involving the probe carrying a PG headgroup (anionic) versus a PC or PE headgroup (zwitterionic) gives an indication of the different extents of segregation of these two lipids induced by interaction with \(\alpha/\beta\)-peptide I versus \(\alpha/\beta\)-peptide II. \(\alpha/\beta\)-Peptide I altered FRET similarly for the NBD probes having either PC, PE or PG headgroups (Fig. 4A), indicating that there was no preferential segregation of PG over PC or PE, either in the presence of PC or PE lipids and therefore, for this peptide, phase boundary defects are not expected to form in the membrane. In contrast, \(\alpha/\beta\)-peptide II was very effective at segregating PG in bilayers composed of DOPE:DOPG (2:1), causing a much greater reduction of

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Fig. 4. Charge segregation studied by FRET between 1 mol% N-Rh-PE and 1 mol% either C6-NBD-PC, C6-NBD-PE or C6-NBD-PG. \(I_o\) is the intensity of fluorescence emission from N-Rh-PE in the absence of peptide and \(I\) the intensity in the presence of the concentration of peptide given in the abscissa. The relative emission intensities at 591 nm (excitation at 465 nm) were plotted as a function of the concentration of peptide added to 50 μM LUVs. Each linear regression line corresponds to a series of determinations as a function of peptide concentration. This peptide titration was repeated once for each set of data giving similar results: (A) \(\alpha/\beta\)-Peptide I with either DOPC:DOPG (2:1) LUVs (top) or DOPE:DOPG (2:1) (bottom). (B) \(\alpha/\beta\)-Peptide II with either DOPC:DOPG (2:1) LUVs (top) or DOPE:DOPG (2:1) (bottom).
FRET with C6-NBD-PG than with C6-NBD-PE (Fig. 4B). This did not occur with bilayers of DOPC:DOPG (2:1).

3.3. Extent of anionic lipid segregation revealed by DSC

For the purpose of this experiment we chose a mixture of zwitterionic and cationic lipids, DPPE and CL that provided a convenient signal in the DSC and was representative of two major classes of lipids found in bacterial membranes. CL provided a more suitable lipid mixture than PG, with reproducible and single component phase transition behavior in the absence of peptide. In contrast, mixtures composed of DPPG and DPPE gave multiple peaks that exhibited variation on repetitive scanning or between replicate samples. CL is also one of the major lipid components of bacterial membranes. At pH 7.4, CL carries a single negative charge [17] as does PG. DPPE in pure form has a gel to liquid crystalline phase transition at neutral pH at 64 °C [18], while the tetraoleoyl form of CL has no observable transition between 0 and 100 °C. A mixture of DPPE:CL (7:3) shows a single, broad transition centered at about 42 °C, indicating a miscibility between these two lipid components (Fig. 5). Addition of 10 mol% of one of the cationic peptides alters the phase transition properties of this lipid mixture and promotes the formation of a component with a transition temperature at about 60 °C, just slightly lower than the phase transition of pure DPPE. This suggests that the cationic peptide preferentially binds to the anionic lipid, CL, leaving a domain highly enriched with DPPE. In the case of α/β-peptide I, the component with a transition temperature of 59.4 °C has an enthalpy of 1.9 kcal/mol, while for peptide II the component at 58.0 °C has an enthalpy of 6.6 kcal/mol. There is a large difference between the two α/β-peptides with regard to the extent to which they cause the formation of this higher melting domain, with α/β-peptide II being more potent than α/β-peptide I.

4. Discussion

In the present study, a mechanism is proposed to explain why α/β-peptide II is more toxic to E. coli than is α/β-peptide I. We suggest that α/β-peptide II can promote phase segregation in membranes composed of both anionic and zwitterionic lipid more potently than can α/β-peptide I. This is shown by fluorescence studies that indicate a phase segregation of anionic and zwitterionic lipid in mixtures of DOPC:DOPG (2:1) (Fig. 4). Furthermore, we more directly demonstrate this segregation with the use of DSC, showing that α/β-peptide II is more potent in removing anionic lipid from a region of the membrane (Fig. 5). This phase segregation results in the formation of a domain highly enriched in PE. This also explains our earlier finding that α/β-peptide II induces the formation of lamellar structures in PE/PG mixtures with a periodicity similar to that found for PE alone and that it causes the adhesion of the inner and outer membranes of E. coli, despite the presence of the intervening peptidoglycan [5]. It thus provides a mechanism to explain why α/β-peptide II is more lytic to LUVs enriched in PE [5] and is also more toxic to E. coli [6]. The effect of α/β-peptide II to induce lipid phase segregation is greater when the zwitterionic lipid is PE than when it is PC (Fig. 4B) [5]. This is different from the tendency of the lipids themselves to be miscible, as it has been observed that PG or CL is more miscible with PE than it is with PC in the absence of any peptide [19]. One of the factors likely to make lipid phase segregation more toxic to organisms with membranes rich in PE or to make liposomes that are rich in PE more lytic is that the formation of a PE-rich membrane domain will itself be damaging to the membrane barrier due to the tendency of PE to form structures with negative curvature.

Why is α/β-peptide II better at sequestering anionic lipids than is α/β-peptide I if both peptides have the same number of charges? Although the design of α/β-peptide I has the charges more clustered together when the peptide forms a 14/15 helix, α/β-peptide II has the charges more clustered along the primary sequence. Thus, α/β-peptide II has two clusters of positive charge at residues 3, 4 and 5 and a second small cluster of the sequential residues 8 and 9. In contrast, α/β-peptide I does not have 2 positively charged residues together in the sequence. Each cationic residue of α/β-peptide I is separated by either two or three other amino acid residues. It is known that several proteins and peptides with clusters of basic amino acid residues along the linear sequence can sequester anionic lipids into domains [20–23]. Our evidence from the FRET studies and DSC indicate that this property extends to α/β-peptide II that similarly has clusters of cationic residues along the sequence.

We have shown previously that α/β-peptide I, but not α/β-peptide II, promotes the formation of a sponge phase in vesicles enriched in PE [5]. Despite this marked change in morphology observed with MLVs, α/β-peptide I does not cause leakage of aqueous contents from LUVs [5]. This difference can now be explained on the basis of the ability of this peptide to promote vesicle-vesicle interaction. The formation of inverted phases from liposomes is initiated by vesicle-vesicle contact [24–28].
Such contact is already present in MLVs because of their morphology. However, this is not the case for LUVs that do not readily convert into inverted phases because of lack of vesicle–vesicle contact. Vesicle–vesicle contact between LUVs can be promoted by peptide-induced aggregation, but this process is not as efficient with α/β-peptide I (Fig. 3A and B). Hence, α/β-peptide I does not readily destabilize LUVs enriched in PE, as is demonstrated by its inability to induce significant leakage in LUVs [5]. Similar arguments can be used to explain why α/β-peptide I is less toxic to E. coli. This peptide is unable to promote nucleation for an inverted or sponge phase despite the proximity of the inner and outer bacterial membrane in the Gram negative bacteria, likely because of the intervening peptidoglycan layer inhibiting contact between these membranes and because of the weak potency of α/β-peptide I to bring bilayers together. This is confirmed by our study of thin sections of E. coli that showed greater separation of inner and outer mitochondrial membranes in the presence of α/β-peptide I [5].

The present study extends our previous work [5] and provides a further understanding of why α/β-peptide II is more toxic to micro-organisms with a high PE content in their membrane as well as for the lack of toxicity of α/β-peptide I with these cells. The work emphasizes the potential importance of the lipid composition of the cell surface in determining selective toxicity of anti-microbial agents.

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