Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of Gram-negative bacteria

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Abstract F12W-magainin 2 preferentially interacted with lipopolysaccharide-containing bilayers, permeabilizing the membranes, compared with lipopolysaccharide-free phosphatidylcholine vesicles. Using this system, we demonstrated for the first time that the magainin peptide forms a helix upon binding to lipopolysaccharide. Incorporation of lipid A into phosphatidylcholine liposomes also enhanced interactions with the peptide. The presence of Mg^{2+}, which nullifies the peptide's antibacterial activity against Gram-negative bacteria, again weakened the interactions between the peptide and lipopolysaccharide-doped bilayers. This system seems to be useful for investigating the molecular details of peptide-lipopolysaccharide interactions.

Key words: Magainin; Lipopolysaccharide; Lipid A; Outer membrane; Membrane permeabilization

1. Introduction

Cationic antimicrobial peptides are an important component of the innate immunity of animals and plants [1]. Many of these peptides, such as magainins [2], cecropins [3] and tachyplesins [4] are considered to kill microorganisms by permeabilizing the cytoplasmic membranes. The observation that enantiomer peptides composed of D-amino acids are equipotent to parent all L-peptides [5,6] suggests that the lipid matrix of the membranes, rather than chiral proteins, is the main target of the peptides. Therefore, a large number of studies have utilized lipid bilayers (liposomes) of various phospholipid compositions as a model of the cytoplasmic membranes to elucidate the molecular mechanisms of membrane specificity and permeabilization [7,8,9].

In contrast, little is known about the interactions of the cationic peptides with negatively-charged cell walls, to which the peptides are expected to bind before reaching the ultimate target. In the case of Gram-negative bacteria, the major component of the cell wall is the outer membrane, which acts as a strong permeability barrier to various antibiotics [10]. The outer leaflet of the outer membrane is mainly built up from polyanionic lipopolysaccharides (LPS), which consist of a polysaccharide moiety and a covalently linked moiety called lipid A. In spite of its importance, relatively few studies have been reported on LPS-peptide interactions. Blazyk and his collaborators used outer membrane-peptidoglycan complexes to study magainin outer membrane interactions [11–13]. They found, using infrared spectroscopy, that the antibiotic peptide perturbs the lipid organization of the membrane. However, this system does not allow estimation of the peptide conformation because it contains many bacterial proteins. Another possibility is the utilization of LPS vesicles. At low concentrations of Mg^{2+}, various rough mutant LPS from *Salmonella minnesota* and *Escherichia coli* form closed vesicles [14]. This model membrane has two problems. First, it is not suitable for optical measurements, such as fluorescence or circular dichroism, because the size is very large ($\sim\mu$m). Second, it is difficult to control the aggregational states of LPS. An increase in the Mg^{2+} concentration leads to precipitation of the vesicles.

In this paper, we utilized phosphatidylcholine (PC) liposomes containing LPS as a model for outer membranes to investigate the interactions between LPS and magainin 2, which is a cationic antimicrobial peptide isolated from the skin of the African clawed frog *Xenopus laevis* and forms a peptide-lipid supramolecular complex pore [15]. We revealed for the first time that the cationic peptide forms a helix upon binding to LPS and lipid A. The effects of the peptide on the barrier property of the bilayers were also examined.

2. Materials and methods

F12W-magainin 2 (GIGKFLHSAKKWGKAFVGEIMNS), an equipotent analog of magainin 2 [16], was synthesized by a standard Fmoc-based solid phase method, as previously described [16]. The purity of the synthesized peptide was determined by analytical HPLC and ion spray mass spectroscopy. Egg yolk-L-α-PC was obtained from Sigma (St. Louis, MO, USA). LPS from *E. coli* O111 (smooth-type) and that from J5 (mutant of O111, Rc-type) were purchased from List Biological Laboratories (Campbell, CA, USA). Synthetic lipid A was a product of Daiichi Pure Chemicals (Tokyo, Japan). Calcein and spectroscopic organic solvents were supplied by Dojindo (Kumamoto, Japan). A 10 mM HEPES/150 mM NaCl/1 mM EDTA (pH 7.4) was prepared with double distilled water.

LPS, lipid A and PC were dissolved in petroleum ether/chloroform/phenol (8/5/2), chloroform/methanol (2/1) and chloroform, respectively. The lipid concentration was determined in triplicate by phosphorus analysis [17]. LPS and PC were mixed in a 1:1 ratio (phosphorus basis) and the lipid A to PC phosphorus ratio was 1:3.

Small unilamellar vesicles (SUVs) for circular dichroism (CD) measurements were produced by a sonication method. The lipid film, after vacuum drying overnight, was hydrated with the buffer and vortex-mixed to produce multilamellar vesicles (MLVs). The MLVs were sonicated to clarity in ice/water under a nitrogen atmosphere. CD spectra of F12W-magainin 2 in the absence or presence of SUVs were recorded on a JASCO J-720 instrument at 37°C using a 1 mm path length quartz cell to minimize the absorbance due to buffer components. The instrumental outputs were calibrated with non-hygroscopic ammonium *d*-camphor-10-sulfonate [18]. Eight scans were averaged for each sample and the averaged blank spectra (the vesicle suspension) were subtracted. The reported spectra are the re-

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sults of averaging at least two independent preparations. The errors were less than 4% at 222 nm.

A binding assay was carried out by CD titration [19]. A peptide solution (3 μM) was titrated while stirring with small aliquots of a LPS (smooth-type)-containing SUV suspension in a 10 mm path length cuvette at 37°C. The ellipticity at 222 nm was recorded in duplicate. The blank values (liposomes only) were subtracted and then, the volume was corrected for dilution. The buffer used was either 10 mM Tris/150 mM NaCl (pH 7.4) or 10 mM Tris/150 mM NaCl/10 mM $MgCl_2$ (pH 7.4).

Dye leakage was measured using large unilamellar vesicles (LUVs). A lipid film was hydrated with a 70 mM calcein solution (pH was adjusted to 7.4 with NaOH) and vortex-mixed to produce MLVs. The suspension was freeze-thawed for five cycles and then successively extruded through polycarbonate filters (a 0.6 μm pore size filter, 5 times; two stacked 0.1 μm pore size filters, 10 times). Vesicles containing calcein were separated from free calcein on a Bio-gel A 1.5 m column. The release of calcein from the LUVs was fluorometrically monitored on a Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 490 nm and emission wavelength of 520 nm at 37°C. The maximum fluorescence intensity corresponding to 100% leakage was determined by the addition of 10% w/v Triton X-100 (20 μl) to 2 ml of the sample. The apparent percentage leakage value was calculated according to $100\times(F-F_0)/(F_t-F_0)$, where F and F_t denote the fluorescence intensity before and after the addition of the detergent, respectively. F_0 represents the fluorescence of intact vesicles.

3. Results

Fig. 1 shows CD spectra of F12W-magainin 2 (30 μM) under various conditions. The peptide assumed an unordered structure in the buffer (trace 1). The presence of PC (1.07 mM) induced only a small change in the spectrum (trace 2). In contrast, the incorporation of smooth-type LPS (trace 5), Rc-type LPS (trace 4) or lipid A (trace 3) into PC induced a helical structure, characterized by double minima at 208–209 and 222 nm. The total phosphorus concentrations for the smooth-type LPS/PC, Rc-type LPS/PC and lipid A/PC systems were 0.74, 0.86 and 1.22 mM, respectively. The spectra are those of the completely membrane bound form because addition of more vesicles did not further change the spectra. The $[\theta]_{222}$ (ellipticity at 222 nm) values for smooth-type LPS, Rc-type LPS and lipid A were $-26\,800\pm700$, $-25\,600\pm500$ and $-23\,600\pm900$ deg cm^2/dmol, respectively.

Binding experiments were performed by CD titration. Fig.

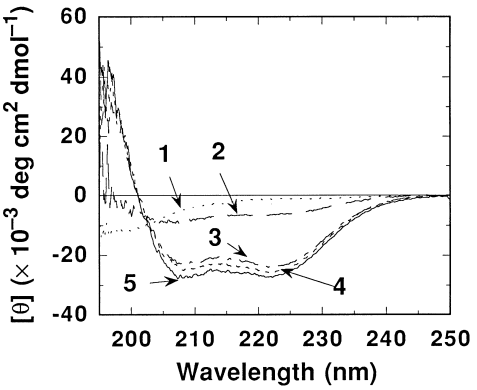

Fig. 1. CD spectra of F12W-magainin 2 at 37°C. Trace 1, in Tris buffer; trace 2, in egg yolk PC SUVs; trace 3, in lipid A/egg yolk PC (1:3, phosphorus basis) SUVs; trace 4, in LPS (Rc)/egg yolk PC (1:1, phosphorus basis) SUVSs; trace 5, smooth-type LPS/egg yolk PC (1:1, phosphorus basis) SUVs.

2A plots the ellipticity at 222 nm as a function of the lipid phosphorus to peptide ratio. In the absence of Mg^{2+} (open circles), the $[\theta]_{222}$ value almost linearly decreased with an increase in the ratio, reaching a plateau where peptide binding was complete. In the presence of 10 mM Mg^{2+} (closed circles), the slope was smaller.

Binding isotherms were calculated from the data in Fig. 2A, assuming that the bound fraction is equal to $([\theta]-[\theta]_{free})/([\theta]_{plateau}-[\theta]_{free})$. Fig. 2B shows the bound peptide per lipid phosphorus, r, against free peptide concentration, $[P]_f$. The affinities were so strong that we could determine only the plateau regions. The binding constants were greater than 10^5 and $2\cdot10^4$ M^{-1} in the absence and presence of the divalent ion, respectively. The saturated r values were approximately 0.04 and 0.02, respectively.

The peptide-induced membrane permeabilization was evaluated using the calcein leakage assay. Fig. 3 exhibits the apparent percentage leakage value, 5 min after the peptide addition as a function of the peptide to lipid phosphorus ratio. The leakage activity of the peptide against PC bilayers was weak. A peptide to phosphorus ratio as high as one was needed to observe a significant extent of dye leakage. The

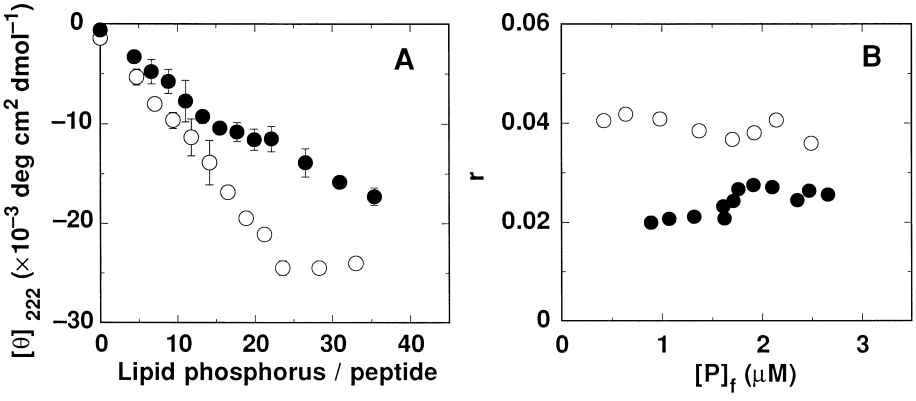

Fig. 2. Binding of F12W-magainin 2 to LPS-containing SUVs at 37°C. (A) CD titration. A 3 μM peptide solution was titrated with SUVs composed of smooth-type LPS/egg yolk PC (1:1, phosphorus basis) while measuring the ellipticity at 222 nm. (B) Binding isotherms. The bound peptide to lipid phosphorus ratio, r, is plotted as a function of the free peptide concentration, $[P]_f$. Open circles, without $MgCl_2$; closed circles, with 10 mM $MgCl_2$.

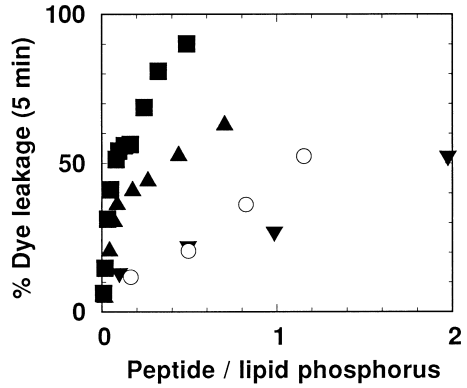

Fig. 3. The percentage leakage of calcein from LUVs of various lipid compositions as a function of the peptide to lipid phosphorus ratio at 37°C. Circles, egg yolk PC; squares, LPS (Rc)/egg yolk PC; triangles, smooth-type LPS/egg yolk PC; inverted triangles, smooth-type LPS/egg yolk PC in the presence of 10 mM $MgCl_2$.

incorporation of smooth-type LPS greatly enhanced the susceptibility of the membrane to the peptide. The amount of the peptide necessary to induce the same extent of leakage was reduced by approximately 1/10. Rc-type LPS with a shorter sugar chain had a greater effect. Fig. 3 also shows that the addition of 10 mM Mg^{2+} completely inhibits the dye-releasing activity of the peptide. The leakage activity was even weaker than that against pure PC bilayers.

4. Discussion

Currently, there are no systems suitable for spectroscopic investigation of molecular details of peptide-LPS interactions. As a consequence, not even the conformation of a peptide bound to LPS has been reported. Regarding the membrane permeability study, LPS/phospholipid asymmetric planar bilayers mimicking bacterial outer membranes have been developed [20]. However, liposomal systems would be highly useful because membrane permeabilization could be directly compared with spectroscopic data [9]. Therefore, we used PC vesicles doped with LPS. Magainin 2, as well as other cationic antimicrobial peptides, is known to only weakly interact with the host zwitterionic lipid [19,21]. LPS is reported to form clusters in a phospholipid matrix [22]. Therefore, we can extract specific interactions between the peptide and the LPS domain.

LPS incorporation induced helix formation (Fig. 1) and the helicity is in the same range as that in acidic phospholipid bilayers (−23 000 to −25 500 deg cm^2/dmol) [23]. The presence of lipid A also triggered helix formation (Fig. 1), suggesting that the negatively-charged carboxyl groups of 2-keto-3-deoxyoctonate residues do not play a crucial role in peptide-LPS interactions. This is probably because the sugar residues are extruded away from the membrane surface and therefore cannot provide hydrophobic interactions between the non-polar face of the amphipathic helix and the hydrocarbon chains.

The affinity of F12W magainin 2 for the smooth-type LPS ($>10^5$ M^{-1}) is comparable to the affinities of magainins for phosphatidylglycerols (10^5–10^6 M^{-1}) [19]. The binding to the LPS saturates at an r value of 0.04 without added Mg^{2+} (Fig. 2B). Assuming that the peptide binds only to the outer surface of the bilayer, the peptide to LPS phosphorus ratio is about 1:6 at saturation. Since the net charge of the peptide is +3–4 at a physiological pH, the low saturation r value means that only a part of the phosphate groups of the lipid A moiety constitute the putative binding site. Mg^{2+} ions appear to competitively block the site (Fig. 2B).

In keeping with the CD data (Fig. 1), LPS addition sensitized peptide-induced membrane permeabilization (Fig. 3). Our system reproduces various observations in peptide-bacteria interactions. First, mutant bacteria having shorter sugar chains are more sensitive to magainin 2 than wild-type strains [13]. The Rc-type LPS more effectively perturbed the barrier property of the membrane compared with the wild-type LPS (Fig. 3). Second, the coexistence of 10 mM Mg^{2+} blocked the bactericidal action of magainin 2 [24]. The addition of the divalent cation made the membrane less susceptible to the peptide than PC liposomes. This is not only because the ion partially inhibits the binding but also probably because Mg^{2+} tightens the lipid packing by crosslinking adjacent phosphate groups of LPS.

In conclusion, we revealed that magainin 2 forms an amphipathic helix upon binding to LPS using LPS-doped phospholipid bilayers. This model system seems to be useful for investigating the interactions between cationic peptides and outer membranes at a molecular level. Phosphatidylethanolamine (PE), which is abundant in bacterial membranes, of course, can be used as the host lipid, although PE itself does not self-assemble to form small closed bilayers. The optimization of experimental conditions, such as the species of the host lipid and the LPS-host lipid ratio, is a subject for future study.

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