Interactions of bovine lactoferricin with acidic phospholipid bilayers and its antimicrobial activity as studied by solid-state NMR

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Abstract

Bovine lactoferricin (LfcinB) is an antimicrobial peptide released by pepsin cleavage of lactoferrin. In this work, the interaction between LfcinB and acidic phospholipid bilayers with the weight percentage of 65% dimyristoylphosphatidylglycerol (DMPG), 10% cardiolipin (CL) and 25% dimyristoylphosphatidylcholine (DMPC) was investigated as a mimic of cell membrane of Staphylococcus aureus by means of quartz crystal microbalance (QCM) and solid-state 31P and 1H NMR spectroscopy. Moreover, we elucidated a molecular mechanism of the antimicrobial activity of LfcinB by means of potassium ion selective electrode (ISE). It turned out that affinity of LfcinB for acidic phospholipid bilayers was higher than that for neutral phospholipid bilayers. It was also revealed that the association constant of LfcinB was larger than that of lactoferrin as a result of QCM measurements. 31P DD-static NMR spectra indicated that LfcinB interacted with acidic phospholipid bilayers and bilayer defects were observed in the bilayer systems because isotropic peaks were clearly appeared. Gel-to-liquid crystalline phase transition temperatures (Tc) in the mixed bilayer systems were determined by measuring the temperature variation of relative intensities of acyl chains in 1H MAS NMR spectra. Tc values of the acidic phospholipid and LfcinB-acidic phospholipid bilayer systems were 21.5 °C and 24.0 °C, respectively. To characterize the bilayer defects, potassium ion permeation across the membrane was observed by ISE measurements. The experimental results suggest that LfcinB caused pores in the acidic phospholipid bilayers. Because these pores lead the permeability across the membrane, the molecular mechanism of the antimicrobial activity could be attributed to the pore formation in the bacterial membrane induced by LfcinB.

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1. Introduction

Bovine lactoferricin (LfcinB) is an antimicrobial peptide which consists of 25-amino acid residues with the amino acid sequence of Phe–Lys–Cys–Arg–Arg5–Trp–Gln–Trp–Arg–Met10–Lys–Lys–Leu–Gly–Ala15–Pro–Ser–Ile–Thr–Cys20–Val–Arg–Arg–Ala–Phe25 forming a disulfide bond between Cys3 and Cys20. That is excised by pepsin digestion in the stomach from the intact 80 kDa bovine milk iron-binding glycoprotein lactoferrin with many immunologically important functions [1]. LfcinB is considerably more active as an antimicrobial peptide than the intact protein [2]. LfcinB is also known to show a lethal effect on a wide range of microorganisms [3]. It has been suggested that the dramatic increase in potency is related to a change in the secondary and tertiary structure of this peptide, changing from a mixed α-helical and β-strand region in the protein to an amphipathic twisted antiparallel β-sheet in the peptide [4]. The solution structure of LfcinB has been determined using 2D 1H NMR spectroscopy. The NMR structure of LfcinB was revealed to be a somewhat distorted antiparallel β-sheet. This contrasts with the X-ray structure of bovine lactoferrin, in which residues 1–13 (of LfcinB) form an α-helix [5]. LfcinB has an extended hydrophobic surface comprised of residues Phe1, Cys3, Trp6, Trp8, Pro16, Ile18, and Cys20. Many hydrophilic and positively charged residues surround the hydrophobic surface, giving LfcinB an amphipathic character. LfcinB bears numerous similarities to a vast number of cationic peptides which exert their antimicrobial activities through membrane disruption. The
structures of these peptides have been well characterized, and models of their membrane-permeabilizing mechanisms have been proposed [6]. The antimicrobial activity of this peptide was analyzed against a number of gram-positive and gram-negative bacteria and was found to inhibit the growth of all the test bacteria at a concentration of 8 μM or less [7].

It is important to characterize the peptide–membrane interaction to understand the antimicrobial activity of peptides. All antimicrobial peptides interact with membrane and tend to be divided into two mechanistic classes such as membrane disruptive and nonmembrane disruptive. Cationic antimicrobial peptides have multiple action on cells ranging from membrane permeability to cell wall and division effects to macromolecular synthesis inhibition. The peptides possess strong selectivity to bacterial membrane and the action responsible for killing bacteria at the minimal effective concentration varies from peptide to peptide and from bacterium to bacterium for a given peptide [8–10]. The primary sequence of LfcinB contains many hydrophobic and positively charged residues, suggesting that it may interact with biological membrane and actually membrane blisters have been observed in bacteria exposed to LfcinB [11].

One mechanism of interaction of cationic antimicrobial peptides with the cell envelope of gram-negative bacteria is discussed as follows [12,13]. Passage across the outer membrane is proposed to occur by self-promoted uptake. Unfolded cationic peptides are associate with the negatively charged surface of the outer membrane and either neutralize the charge over a patch of the outer membrane, creating cracks through which the peptide can cross the outer membrane, or actually bind to the divalent cation binding sites on the surface and disrupt the membrane. Once the peptide has transited the outer membrane, it will bind to the negatively charged surface of the cytoplasmic membrane, created by the head groups of phosphatidylglycerol and cardiolipin (CL), and the amphipathic peptide will insert into the membrane interface. It is not known at which point in this process the peptide actually folds into its amphipathic structure. Many peptide molecules will insert into the membrane interface and then either aggregate into a micelle-like complex which spans the membrane or flip-flop across the membrane under the influence of the large transmembrane electrical potential gradient. The micelle-like aggregates are proposed to have water associated with them, and this provides channels for the movement of ions across the membrane and possibly leakage of larger water-soluble molecules. These aggregates would be variable in size and lifetime and will dissociate into monomers that may be disposed at either side of the membrane. One of these types of actions with peptides is proposed as a troidal model in the magainine–lipid bilayer systems [14–16]. In the membrane disruptive peptides, they often form α-helix. Three mechanistic models, the “barrel stave” [17], “micellar aggregate” [12] and “carpet” [18] models are proposed to explain membrane disruptive properties of peptides. Peptide insertion and properties of the aggregation have been observed in melittin–lecithin bilayer systems [19–21] and other antimicrobial peptides [22–26]. It is of equal importance to determine the structure and orientation of peptide bound to membrane if one wants to understand the action of peptides on membrane on a molecular basis [27–30].

It is thought that LfcinB has specificity of phospholipid bilayers to a particular cell membrane of bacteria rather than that of eukaryotic membrane. Actually dimeristoylphosphatidylglycerol (DMPG) is the major component of gram-positive bacteria, while dimysteoylphosphatidylethanolamine (DMPE) is the major component of gram-negative bacteria. DMPG rich lipid bilayers can be expected to have large affinity with a peptide with basic amino acids. On the other hand, DMPE rich lipid bilayers are known to show non-lamellar phase such as hexagonal, cubic or inverted micelles [31]. We, therefore, investigated the specific interaction of LfcinB with acidic phospholipid bilayers consisting of three phospholipids with the weight percentage of 65% DMPG, 10% CL and 25% dimeristoylphosphatidylethanolamine (DMPC) as a mimic of cell membrane of Staphylococcus aureus by means of quartz crystal microbalance (QCM) technique, solid-state 13P and 1H NMR spectroscopy.

2. Materials and methods

2.1. Peptides

LfcinB was synthesized by means of a solid phase method using an Applied Biosystems 431A peptide synthesizer with HMP resin (Applied Biosystems, Inc., Foster City, California). After removing protecting groups and cleavage from the resin, the synthesized peptides were purified using a Waters 600E high-performance liquid chromatography (HPLC) equipped with a Wako Navi C18-5 reversed-phase column. We use a mixture of Milli-Q water and acetonitrile containing 0.05% TFA as a mobile phase. The disulfide bridge between Cys3 and cys20 was formed by air oxidation at pH 8.5 with diluted concentration of 1 mg/mL. The reaction was stopped by adding acetic acid solution and then the crude LfcinB was purified by HPLC. LfcinB was lyophilized directly from aqueous solution after HPLC purification. The production of LfcinB was identified by mass spectrometry using a PerSeptive Biosystems Voyager MALDI-TOF/MS. In the MALDI-TOF/MS experiments, LfcinB was dissolved in Milli-Q water containing 0.05% TFA at a concentration of 5 nmol/mL.

2.2. Lipid dispersions for NMR measurements

DMPC, DMPG and CL were purchased from Sigma and used without further purification. CL was dissolved in ethanol (4.8 mg CL/1 mL ethanol). Acidic phospholipid bilayers with the weight percentage of 65% DMPG, 10% CL and 25% DMPC as a mimic of cell membrane of Staphylococcus aureus were prepared in this study: 50 mg of a mixture of 65% DMPG, 10% CL and 25% DMPC was dissolved in the solvent with 4.5 mL chloroform and 4.5 mL methanol. The solvent was subsequently evaporated in vacuo, followed by hydration with 500 μL Tris buffer (20 mM Tris, 100 mM NaCl and pH 7.5). A freeze–thaw cycle was repeated 10 times, followed by incubating the samples for overnight at 40 °C. These preparations were used for 31P NMR measurements. For 1H NMR measurements, the preparations were lyophilized and hydrated with deuterium oxide. When LfcinB was mixed with the lipids to give lipid–LfcinB dispersion samples, two types of dispersion samples were prepared. First, after the lipid dispersion was prepared, LfcinB was added to it and this sample is called “LfcinB added bilayers”. Second, LfcinB was resolved with the lipid in the mixed solvent of chloroform and methanol, followed by evaporation in vacuo. Consequently, this sample was hydrated and incubated overnight. This sample is called “LfcinB incorporated bilayers”.

2.3. QCM measurements

The association constants (Ka) of LfcinB and lactoferrin binding to various phospholipid bilayers were measured by means of QCM (AT cut shear mode, 27 MHz) using an AffinisQ4 (Initium, Tokyo, Japan). A frequency decrease of
phospholipid bilayers as determined by QCM

1 Hz corresponding to 0.61 ± 0.1 ng cm⁻² on the electrode can be detected by a 27 MHz QCM [32]. Acidic phospholipids were immobilized on an Au electrode of QCM as multibilayer films. A chloroform and ethanol solution of various phospholipids was cast on electrode of QCM. This film was dried in the N₂ stream and aged in Tris buffer (20 mM Tris, 100 mM NaCl and pH 7.5) at 30 °C for 1 h. After the immobilization of acidic phospholipids on the Au electrode of QCM was achieved, the frequency changes of the QCM responding to the addition of LfcinB and lactoferrin was recorded for about 100 s until the equilibrium was reached in Tris buffer at 25 °C.

As the injected concentrations of the LfcinB and lactoferrin were increased in the solution, the association constants (Kₐ) can be calculated from the binding amounts (Δm) and concentrations of LfcinB and lactoferrin using the equation as follows,

\[
\frac{[\text{guest}]_m}{\Delta m} = \frac{[\text{guest}]_0}{\Delta m_{\text{max}}} + \frac{1}{\Delta m_{\text{max}}K_a}
\]

where [guest]₀ is the initial concentration of LfcinB or lactoferrin and Δmₘₐₓ is the maximum binding amount. Because the electrode is vigorously stirred, only strong binding molecules with lipid bilayers can contribute to the increase of Δm. When the plot of [guest]₀/Δm against [guest]₀ gives a simple straight line, the association constant (Kₐ) was calculated from the slope and the intercept when [guest]₀ = 0 in the plot.

2.4. NMR measurements

³¹P and ¹H NMR spectra were recorded on a Chemagnetics CMX 400 Infinity NMR spectrometer at the ³¹P and ¹H resonance frequencies of 161.1 and 398.1 MHz, respectively, under static or magic angle spinning (MAS) conditions with DD method (high-power dipolar decoupling). ³¹P and ¹H chemical shift values were referred to those of 85% H₃PO₄ and tetramethylsilane (TMS), respectively. The numbers of transients for ³¹P DD-static and ¹H MAS NMR measurements were 1000 and 100, respectively. Each sample was allowed to equilibrate for 30 min before starting the NMR measurements. We use a glass sample tube in ³¹P DD-static NMR measurements and a zirconia one in ¹H MAS NMR measurements.

2.5. Measurements of potassium ion permeation by ion selective electrode

We measured the time course of concentration variation of K⁺ after adding LfcinB to acidic phospholipid bilayer vesicles by an ion meter LAB F-23 (Horiba, Kyoto, Japan) together with potassium ion selective electrode (ISE). Large unilamellar vesicles (LUV) were prepared with acidic phospholipid bilayers in Tris buffer (20 mM Tris, 100 mM KCl and pH 7.5) by using extrusion and filtration with 800 nm micro pore filters. After preparing LUV, the dispersions of 0.2 ml were diluted with Tris buffer (20 mM Tris, 100 mM NaCl and pH 7.5) of 25 ml, so that K⁺ ions in Tris buffer (20 mM Tris, 100 mM KCl and pH 7.5) were trapped inside the vesicles. Concentration of lipid dispersion was 125 times lower than that for NMR measurements, leading to less aggregation among the vesicles. Concentration of K⁺ outside the vesicles was measured with ISE. LfcinB solution of 0.1 ml (5 mM) was added to the diluted LUV dispersed solution.

3. Results and discussion

3.1. Interaction of LfcinB and lactoferrin with acidic phospholipid bilayers as determined by QCM

Fig. 1 shows the plots of association constants of LfcinB and lactoferrin against the ratio of DMPG in the DMPC and DMPG mixed membrane. The association constants were determined by QCM. The association constants of LfcinB increased with the proportion of DMPG in the DMPC and DMPG mixed membrane. It is noticed that the Kₐ values start steeply increasing from the DMPG percentage of 60%. The association constants of lactoferrin, however, didn’t increase at any proportion of DMPG/(DMPC+DMPG). These results indicate that affinity of LfcinB for acidic phospholipid bilayers is higher than that for neutral ones. It also indicates that LfcinB was associated with acidic phospholipid bilayers more strongly than lactoferrin. Moreover, we determined the association constants of LfcinB- and lactoferrin-acidic phospholipid bilayer systems as bacteria mimic membranes as summarized in Table 1. It turned out that the association constant of LfcinB with acidic phospholipids was 16 fold larger than that of lactoferrin. This large affinity of LfcinB for acidic phospholipids may correlate with the large potency of LfcinB for killing bacteria as compared with lactoferrin as a precursor protein. It is important to point out that the association constants start steeply increasing from the DMPG ratio of 60%. This result indicates that LfcinB interacts specifically with the cell membrane of bacteria rather than eukaryotic membrane.

3.2. ³¹P NMR spectra of LfcinB-acidic phospholipid bilayers

Fig. 2 shows the temperature variation of ³¹P NMR spectra of acidic phospholipid bilayers (a) and LfcinB–phospholipid bilayer systems (b, c). Acidic phospholipid bilayers were hydrated with Tris buffer (20 mM Tris, 100 mM NaCl and pH 7.5). After the measurements of ³¹P NMR spectra of the acidic phospholipid bilayers, LfcinB in Tris buffer solution was added to the same acidic phospholipid bilayer sample (Fig. 2b). It was already revealed by QCM that LfcinB was strongly associated with acidic phospholipid bilayers. Molar ratio of peptide to acidic phospholipid bilayers was 1:20 throughout these experiments. Immediately after the sample was placed in the magnetic field, the ³¹P NMR spectra were recorded at 40 °C. As soon as LfcinB was added to the acidic phospholipid bilayers, small isotropic signals were appeared at 0 ppm (Fig. 2b). Spectral area of the isotropic component was estimated to be about 2% as compared with the whole area of the spectrum at 40 °C for LfcinB added sample. When hydration was performed after preparing the mixed film of LfcinB and acidic lipids from organic solvents (LfcinB
incorporated lipid sample), larger isotropic peaks at 0 ppm with the isotropic component of 5.2% appeared as shown in Fig. 2c at 40 °C. Since this ratio is similar to the 5% of the LfcinB out of lipid molecules, the formation of the isotropic component may correlate with the presence of LfcinB in the membrane. However, these isotropic peaks disappeared at a lower temperature than 30 °C. These results indicate that defects in lipid bilayers such as non-lamellar lipid phase may be formed in the presence of LfcinB to cause isotropic components in the acidic phospholipid bilayers at 40 °C. These defects may lead the permeability in the membrane which is related to the antimicrobial activity. It is noticed that the spectral pattern of LfcinB-acidic phospholipid bilayer systems at each temperature was changed to the larger δ// value than that of acidic phospholipid (Table 2a and b). These results indicate that the anisotropy of 31P NMR powder pattern increased by adding LfcinB, implying that LfcinB reduced mobility or changed the direction in the head group of acidic phospholipid bilayers because the interaction of LfcinB with lipid head groups are considered.

Large difference was seen in 31P NMR spectra between LfcinB added- and LfcinB incorporated-acidic phospholipid bilayer systems. When LfcinB was added to the acidic phospholipid bilayer systems, pronounced aggregation of the vesicles was observed. This strong interaction between vesicles may be originated the amphipathic nature of LfcinB bound to membranes. Heavy aggregations are caused by the hydrophobic interaction of LfcinB whose hydrophobic side is exposed to the outside vesicles. This may prevent for LfcinB to insert to the membrane. On the other hand, incorporated LfcinB can be deeply inserted into the inner bilayers of vesicles and shows pronounced reduction of 31P anisotropy and increase of isotropic peaks.

3.3. Gel-to-liquid crystalline phase transition temperatures (Tc) determined by 1H MAS NMR spectra

To verify specific interaction of LfcinB with acidic phospholipid bilayers, gel-to-liquid crystalline phase transition temperatures (Tc) were determined by measuring the temperature variation of relative intensities of acyl chains in 1H MAS NMR spectra (Fig. 3). Large intensity variations were observed particularly in the 1H MAS NMR spectra of methylene protons (indicated by arrow) because of the change of the mobility of acyl chains. This change of mobility is strongly correlated to the phase properties of gel-to-liquid crystalline phase transitions because the mobility of molecules changes significantly across the phase transition temperature. This causes the changes of 1H NMR line-widths. It is clearly shown in the plots of intensity variations against temperatures (Fig. 4) that the phase transition of pure DMPC lipid bilayer occurred in the narrow temperature range. On the other hand, mixed acidic phospholipids show gradual change of the mobility against the temperatures. Tc values of acidic phospholipid and LfcinB-acidic phospholipid bilayer systems were determined to be 21.5 °C and 24.0 °C, respectively, by taking the mid points of the curve of the intensity variations. Tc values of DMPC were also measured to

Table 1

<table>
<thead>
<tr>
<th>Association constants (M⁻¹)</th>
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<tbody>
<tr>
<td>LfcinB-acidic phospholipid bilayer systems</td>
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<td>Lactoferrin-acidic phospholipid bilayer systems</td>
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Table 2

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<tr>
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<th>δ⊥</th>
<th>δ∥</th>
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<tr>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
</tr>
<tr>
<td>40 °C</td>
<td>−11.45</td>
<td>−11.46</td>
</tr>
<tr>
<td>30 °C</td>
<td>−11.71</td>
<td>−11.71</td>
</tr>
<tr>
<td>20 °C</td>
<td>−11.98</td>
<td>−11.95</td>
</tr>
<tr>
<td>10 °C</td>
<td>−12.02</td>
<td>−12.73</td>
</tr>
<tr>
<td>0 °C</td>
<td>−7.89</td>
<td>−11.82</td>
</tr>
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a The precision is ±0.15 ppm.  
b The precision is ±0.50 ppm.
be 23.0 °C that agrees well with the reported Tc value of DMPC
[33]. It is revealed that the interaction among lipids in the cell
membrane of Staphylococcus aureus became weaker than
DMPC phospholipids bilayers. This result can be interpreted
that excess negative charge of DMPG and CL with four acyl
chains weaken the interactions among lipids of cell membrane
of Staphylococcus aureus because of the electron repulsive
interaction. It was therefore proved that the doubly charged CL
may cause disruption of acidic phospholipid bilayers. On the
other hand, when LfcinB was added to acidic phospholipid
bilayers, Tc value of LfcinB-acidic phospholipid bilayer systems
increased by 2.5 °C. This can be attributed that the positive charge
of LfcinB neutralizes the surface charge of cell membrane
and consequently the interaction among lipids becomes
strengthened. It, therefore, turned out that LfcinB played an important role to make acidic phospholipid bilayers rigid. This trend agrees with the increase of the anisotropy of
31P NMR pattern at 40 °C for LfcinB-acidic membrane systems
as compared with the acidic membrane systems without LfcinB.

3.4. Pore formation of LfcinB into acidic phospholipid bilayers
as disclosed by potassium ion selective electrode

31P NMR results show that defects in lipid bilayers may be
formed because isotropic peak appeared (Fig. 2b) in the
powder pattern at 40 °C. To characterize the properties of the
bilayer defect, we measured the time course of concentration
variation of K+ after adding LfcinB to acidic phospholipid vesicles entrapped K+ ions by using potassium ISE. Fig. 5
shows that potassium ion permeation across the membrane was observed when LfcinB was added at the arrow position in
Fig. 5 at 40 °C. Although gradual ion permeation was observed in the acidic vesicles, further increase of ion
permeation was appeared by additions of LfcinB. Similar
ion permeation effects were observed in dynorphin–DMPC
[34] and melittin–DMPC systems [21] by using potassium
ISE, in which Triton x-100 solution was added to the bilayer
systems to dissolve the lipid in the buffer. In these cases, the
increase of the K+ concentration was also observed. There-
fore, one of the possible states of acidic phospholipids with
LfcinB is to form pores such as troidal pores since LfcinB
may not dissolve the lipids to be permeable the potassium
ions. Ion permeation was saturated after an addition of LfcinB
solutions because the concentration equilibrium between
inside and outside was achieved. Since the isotropic
component in 31P NMR exists, the possibility of the formation
of cubic phase or other non-lameller phase cannot be ruled
out. These results, however, clearly indicate that the acidic phospholipid bilayer defects induced by LfcinB cause ion
permeation across the membrane.

4. Conclusions

It is clearly demonstrated that affinity of LfcinB for acidic phospholipid bilayers are higher than neutral phospholipid bilayers. This result explains that LfcinB selectively interacts to the bacteria membrane rather than eukaryotic membrane. It was
also revealed that LfcinB caused defects in the acidic membranes and the defects turned out to be pores in the
membrane. Because these pores lead the permeability of potassium ions across the membrane as observed by ion
selective electrode, the molecular mechanism of the antimicrobial activity of LfcinB could be attributed to the pore formation induced by LfcinB. It is stressed that a combined study of solid-state $^{31}$P DD-static and $^1$H MAS NMR seperately provides dynamic properties of polar group and acyl chains in lipid bilayers, respectively.

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