Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1758 (2006) 1523–1528
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bbamem

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

Masako Umeyama [a], Atsushi Kira [b], Katsuyuki Nishimura [a], Akira Naito [a,*]

[a] *Graduate School of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan*
[b] *Research and Development Division, ULVAC, Inc. 2500 Hagizono, Chigasaki, Kanagawa 253-8543, Japan*

Received 5 February 2006; received in revised form 10 June 2006; accepted 12 June 2006
Available online 18 June 2006

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 ^{31}P and ^{1}H 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. ^{31}P 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 ^{1}H 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.

Keywords: Lactoferricin; Antimicrobial peptide; Acidic phospholipid bilayers; Pore formation; Solid-state ^{31}P and ^{1}H NMR; Gel-to-liquid crystalline phase transition temperature

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–Arg^{5}–Trp–Gln–Trp–Arg–Met^{10}–Lys–Lys–Leu–Gly–Ala^{15}–Pro–Ser–Ile–Thr–Cys^{20}–Val–Arg–Arg–Ala–Phe^{25} 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 ^{1}H 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

* Corresponding author. Tel.: +81 45 339 4232; fax: +81 45 339 4251.
E-mail address: naito@ynu.ac.jp (A. Naito).

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.06.014

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 dimyristoylphosphatidylglycerol (DMPG) is the major component of gram-positive bacteria, while dimyristoylphosphatidylethanolamine (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-lameller phase such as hexagonal, cubic or inversed 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% dimyristoylphosphatidylcholine (DMPC) as a mimic of cell membrane of *Staphylococcus aureus* by means of quartz crystal microbalance (QCM) technique, solid-state ^{31}P and ^{1}H 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 ^{31}P NMR measurements. For ^{1}H 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 (K_a) of LfcinB and lactoferrin binding to various phospholipid bilayers were measured by means of QCM (AT cut shear mode, 27 MHz) using an AffinixQ4 (Initium, Tokyo, Japan). A frequency decrease of