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Membrane curvature stress and antibacterial activity of lactoferricin derivatives

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Abstract

We have studied correlation of non-lamellar phase formation and antimicrobial activity of two cationic amphipathic peptides, termed VS1-13 and VS1-24 derived from a fragment (LF11) of human lactoferricin on *Escherichia coli* total lipid extracts. Compared to LF11, VS1-13 exhibits minor, but VS1-24 significantly higher antimicrobial activity. X-ray experiments demonstrated that only VS1-24 decreased the onset of cubic phase formation of dispersions of *E. coli* lipid extracts, significantly, down to physiological relevant temperatures. Cubic structures were identified to belong to the space groups Pn3m and Im3m. Formation of latter is enhanced in the presence of VS1-24. Additionally, the presence of this peptide caused membrane thinning in the fluid phase, which may promote cubic phase formation. VS1-24 containing a larger hydrophobic volume at the N-terminus than its less active counterpart VS1-13 seems to increase curvature stress in the bilayer and alter the behaviour of the membrane significantly enhancing disruption.

Keywords: Membrane curvature stress; Antimicrobial peptide; Lactoferricin; *E. coli*; Model membrane

Since increasing numbers of bacterial strains are getting resistant to conventional antibiotics, there rises an urgent need for new antimicrobial agents [1,2]. One strategy is based on host defence peptides that can be found in every organism including humans [3]. Most of these peptides are supposed to kill the target organisms by membrane permeabilization [4]. We have studied the antimicrobial peptide LF11 [5] and its derivatives. LF11, known for its antimicrobial activity, is derived from the pepsin cleavage product of human lactoferricin [6,7] and exhibits an amphipathic structure, which is formed only upon their contact with membranes, whereas it possesses no defined 3D structure in solution [8,9]. These peptides are cationic, a property that is believed to form the basis for their selectivity against negatively charged bacterial membranes [4,6,10]. Moreover, it is known that for antimicrobial activity a threshold value of hydrophobicity is required [11–13]. However, a certain balance between charge and hydrophobicity is needed to prevent loss of target cell selectivity.

The low activity of LF11 against a number of Gram-positive and Gram-negative reference strains [5,14] indicated already the need for strengthening membrane interaction by variation of the LF11 amino acid sequence. Therefore, we have performed structure–activity relationship studies on LF11 and more than 100 derivatives of this peptide containing single point mutations and/or *N*-acylations, resulting in peptides with up to 100-fold improvement of antimicrobial activity (manuscript in preparation). Here, we report on biophysical studies of two peptides, VS1-13 and VS1-24, derived from LF11 that showed increased activity towards *Escherichia coli* (see Table 1). Studies on the interaction of the antimicrobial peptide gramicidin S with *E. coli* lipid extracts demonstrated that this cyclic peptide promotes the formation of inverted cubic structures [15]. It was proposed that this effect may be an important aspect of membrane permeabilization and/or rupture, which appears to be the

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Table 1
Primary structure of the parent peptide LF11 and its derivatives VS1-13 and VS1-24 and their minimal inhibitory concentration (MIC) towards *E. coli* as well as haemolytic activity towards human red blood cells (RBCs)

Peptide	Amino acid sequence														MIC [μg/ml][a] *E. coli* ATCC 25922	% Lysis of 2.5% RBCs[b] (peptide 500 μg/ml)
LF11			F	Q	W	Q	R	N	I	R	K	V	R	$-NH_2$	>250	0
VS1-13			F		W	Q	R	N	I	R	I	R	R	$-NH_2$	125	0
VS1-24	P	F	F		W		R		I	R	I	R	R	$-NH_2$	8	10

[a] MICs were determined as peptide concentration resulting in less than 2% growth following an overnight incubation in Mueller Hinton medium at 37 °C in the presence of 5×10^5 CFU/ml.
[b] Percent lysis were calculated following 1 h incubation at 37 °C in PBS using 1% triton as 100% lysis and PBS as 0% lysis.

mechanism by which this peptide kills bacteria. Thus, this study focused on the ability of VS1-13 and VS1-24 to promote non-lamellar structures and on its correlation with their biological activity. In order to mimic the target of the peptides, membrane model systems composed of *E. coli* lipid extracts were used.

Materials and methods

Lipids and peptides. Escherichia coli total membrane lipid extract (58% phosphatidylethanolamine (PE), 15% phosphatidylglycerol (PG), 10% cardiolipin (CL) and 17% other) was obtained from Avanti Polar Lipids, Inc. (USA) and used without further purification. Stock solutions were prepared in $CHCl_3/CH_3OH$ (9:1, v/v) and stored at −18 °C.

The peptides, VS1-13 (FWQRNIRIRR-NH_2, MW = 1444 g/mol) and VS1-24 (PFFWRIRIRR-NH_2, MW = 1446 g/mol) were purchased from NeoMPS, Inc. (San Diego, CA, USA). The purities were >75% as determined by RP-HPLC. Peptides were dissolved in phosphate buffered saline (PBS, 20 mM NaP_i, 130 mM NaCl, pH 7.4) at a concentration of 4 mg/ml before each experiment.

Assays for antimicrobial and hemolytic activity. Antimicrobial activity of the peptides towards *E. coli* ATCC 25922 was tested using susceptibility microdilution assays according to NCCLS (National Committee for Clinical Laboratory Standards) approved guidelines and hemolytic activity towards human red blood cells was determined as described elsewhere [5]. All assays were performed in duplicate and three times.

Preparation of liposomes. Aqueous dispersions of *E. coli* total lipid extract of 5 wt% in PBS-buffer in the presence (lipid-to-peptide molar ratio of 25:1) and absence of peptides were prepared as described by Staudegger et al. [15]. The fully hydrated samples were stored at room temperature until measurement.

Small-angle X-ray scattering (SAXS). X-ray diffractograms were recorded with a SWAX-camera (HECUS X-ray Systems, Graz, Austria), which was mounted on a sealed-tube Seifert generator (Ahrensburg, Germany) operating at 2 kW. The X-ray beam was filtered for CuK_α-radiation ($\lambda = 1.54$ Å) using a Ni foil and a pulse-height discriminator, built into the detection system. The scattered intensity was recorded with a linear position-sensitive detector (HECUS X-ray Systems, Graz, Austria) in the small-angle regime of 5×10^{-3} Å$^{-1} < q < 0.5$ Å^{-1}, where $q = 4\ \pi\ \sin(\theta)/\lambda$ is the modulus of the scattering wave vector. Calibration in the small-angle region was performed with a silver stearate standard. Samples were loaded into thin-walled quartz capillaries that were thermally equilibrated for 10 min before initiating data acquisition. Temperature was controlled with an accuracy of 0.1 °C with a programmable Peltier unit. Scattering patterns were recorded at each temperature for 7200 s.

Results and discussion

Biological activity data and properties of LF11-derivatives

Antimicrobial and haemolytic activity of the three peptides LF11, VS1-13, and VS1-24 are summarized in Table 1, clearly demonstrating that concerted variations of amino acids of the parent peptide sequence of LF11 can significantly affect the biological activity. The LF11 derived peptide VS1-13 exhibits a strengthening of the hydrophobic N-terminus by deletion of an N-terminal glutamine (Q). In addition, an arginine (R) was added at the C-terminus. These features resulted in a decrease of the minimal inhibitory concentration (MIC) for *E. coli* (ATCC 25922) without inducing haemolytic activity. Although, the MIC value for VS1-13 is lowered as compared to its parent peptide LF11, it is still a weakly active peptide. In contrast, the MIC value determined for VS1-24 is in the range of a highly potent antimicrobial peptide [16] displaying a MIC value of 8 μg/ml, which corresponds to a ~30-fold decrease as compared to LF11. This can be attributed to the further strengthening of the hydrophobic N-terminus by addition of proline (P) and phenylalanine (F), as well as deletion of polar amino acids in the core of the peptide, and follows the general hypothesis of a balanced charged and hydrophobic amino acid distribution. The former is necessary to interact specifically with negatively charged membrane lipids such as phosphatidylglycerol (PG), cardiolipin (CL) and lipopolysaccharides (LPS), the charged lipid components of *E. coli* inner and/or outer membrane, and the latter to enhance penetration of the peptide into the hydrophobic part of the lipid bilayer to disrupt membrane integrity. However, too hydrophobic peptides often also permeate membranes composed of neutral lipids like erythrocytes [5], which to some extent is the case for VS1-24 exhibiting minor haemolytic activity (Table 1). Nevertheless, this activity is negligible when compared to the MIC value for *E. coli*, still yielding a suitable therapeutic index.

X-ray studies on E. coli membrane mimics in the lamellar fluid state

Experiments were performed in the biologically relevant fluid phase, where the hydrocarbon chains of the lipids are in a melted state. Small angle X-ray scattering (SAXS) data of aqueous dispersions of *E. coli* membrane total lipid extracts at 25 °C showed a diffuse scattering with a broad intense side maximum around 70 Å ($q \sim 0.09$ Å^{-1}) and a very minor one around 22 Å ($q \sim 0.29$ Å^{-1}) (Fig. 1). This pattern is typical for particle scattering as known for unilamellar vesicles or uncorrelated bilayers. Background corrected SAXS patterns were analyzed in terms of a global