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Structural and biophysical characterization of an antimicrobial peptide chimera comprised of lactoferricin and lactoferrampin

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ABSTRACT

Lactoferricin and lactoferrampin are two antimicrobial peptides found in the N-terminal lobe of bovine lactoferrin with broad spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacteria as well as *Candida albicans*. A heterodimer comprised of lactoferrampin joined to a fragment of lactoferricin was recently reported in which these two peptides were joined at their C-termini through the two amino groups of a single Lys residue (Bolscher et al., 2009, Biochimie 91(1):123–132). This hybrid peptide, termed LFchimera, has significantly higher antimicrobial activity compared to the individual peptides or an equimolar mixture of the two. In this work, the underlying mechanism behind the increased antibacterial activity of LFchimera was investigated. Differential scanning calorimetry studies demonstrated that all the peptides influenced the thermotropic phase behaviour of anionic phospholipid suspensions. Calcein leakage and vesicle fusion experiments with anionic liposomes revealed that LFchimera had enhanced membrane perturbing properties compared to the individual peptides. Peptide structures were evaluated using circular dichroism and NMR spectroscopy to gain insight into the structural features of LFchimera that contribute to the increased antimicrobial activity. The NMR solution structure, determined in a miscible co-solvent mixture of chloroform, methanol and water, revealed that the Lys linkage increased the helical content in LFchimera compared to the individual peptides, but it did not fix the relative orientations of lactoferricin and lactoferrampin with respect to each other. The structure of LFchimera provides insight into the conformation of this peptide in a membranous environment and improves our understanding of its antimicrobial mechanism of action.

1. Introduction

The majority of biophysical studies concerning antimicrobial peptides published to date focus on a variety of experiments performed on highly purified peptide samples in an effort to characterize the antimicrobial mode of action of a single peptide [1]. A recent trend has seen the emergence of reports that focus on the combined effects of antimicrobial peptides wherein a mixture of peptides, administered together, cause a more substantial antimicrobial effect [2–4]. The concept of synergy between antimicrobial peptides is rapidly gaining momentum as researchers seek to enhance the antimicrobial activity and effectiveness of peptide sequences [5]. The combined effect of multiple antimicrobial peptides has inspired the generation of unique hybrid antimicrobial peptides in which peptides are covalently linked to each other to generate molecules with enhanced antimicrobial activity. An example of such a hybrid peptide is the disulphide-linked magainin-PGLa heterodimer [6] for which we recently published the micelle bound structure [7]. In the present work, the structure and membrane perturbing properties of a novel hybrid peptide comprised of two antimicrobial sequences obtained from the intact bovine lactoferrin protein is evaluated and compared to each of the component peptides. A unique feature of this peptide chimera is that the two peptides are covalently joined at their C-termini through the two amino groups of a single lysine residue.

Lactoferrin is an 80 kDa iron binding protein found in the secretory fluids of mammals. In addition to its well-documented ability to bind iron, which contributes directly to its antibacterial properties by sequestering iron required for bacterial growth [8, 9], lactoferrin also possesses a wide range of biological functions (unrelated to iron binding) including antibacterial, antiviral, anticancer and immunomodulatory activities [10–12]. Of particular interest to antimicrobial peptide researchers is the cationic N-terminal lobe of bovine lactoferrin which includes two antimicrobial sequences, lactoferricin and lactoferrampin (Fig. 1).

Abbreviations: COSY, Correlation spectroscopy; DPPG, 1,2-dihexadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DSC, Differential scanning calorimetry; ePE, egg derived phosphatidylethanolamine; ePG, egg derived phosphatidylglycerol; LFampB, Bovine lactoferrampin; LFchimera, Lys linked LFcin17–30 and LFampB heterodimer; LFcin, Lactoferricin; LUV, Large unilamellar vesicle; NMR, Nuclear magnetic resonance; NOESY, Nuclear Overhauser spectroscopy; PLE, *E. coli* polar lipid extract; RMSD, Root mean squared deviation; SDS, Sodium dodecyl sulphate; TOCSY, Total correlation spectroscopy

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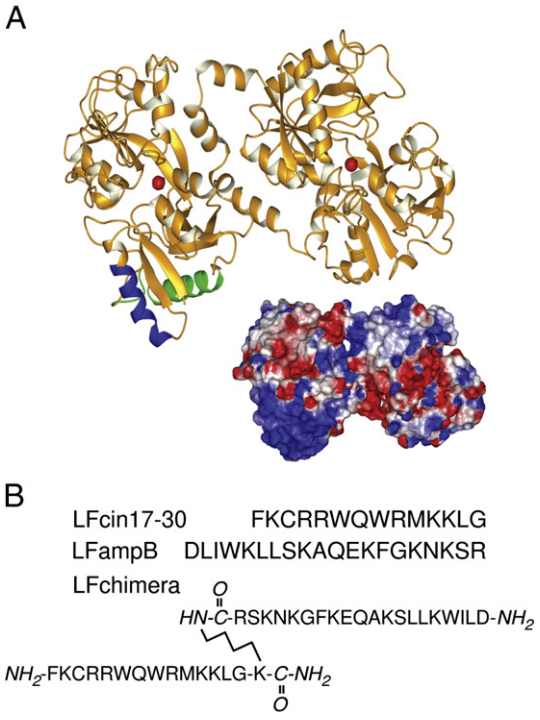

Fig. 1. Crystal structure of bovine lactoferrin (PDB ID = 1BLF, A). Each lobe of lactoferrin is capable of binding to one iron atom, shown in red. Bovine lactoferrin is the source of two antimicrobial peptides, lactoferricin and lactoferrampin. LFcin17–30 (blue) and LFampB (green) are found in the highly cationic N-terminal lobe of bovine lactoferrin as seen in the space filling model of the protein with positively charged regions coloured in blue, negatively charged regions in red and neutral regions appearing white. The sequences of the two peptides, as well as the unique sequence of LFchimera, in which the two peptides are joined through a single Lys residue, are shown in (B). Joining these two peptides through a single lysine residue makes it possible for these sequences to maintain a relative orientation with respect to each other, similar to that seen in the crystal structure of bovine lactoferrin.

Lactoferricin (LFcin), is an antimicrobial peptide that has been extensively studied by several groups, including our own. LFcins are released from lactoferrin by the proteolytic activity of pepsin in the gut [13–15]. These polypeptides display significantly higher antibacterial activity compared to the intact lactoferrin protein and considerable effort has gone into elucidating their mechanism of action [16]. Bovine LFcin corresponds to residues 17–41 of lactoferrin and it forms an amphipathic β-sheet hairpin structure in aqueous solution [17] that is distinct from the α-helical conformation seen in the corresponding sequence in the crystal structure of bovine lactoferrin [18]. Shorter versions of bovine LFcin retain much of the antimicrobial activity of the native peptide and these truncated forms have provided detailed structural, functional and biophysical insights into the interaction between LFcin and lipid bilayers or bacterial cells [19–24].

Bovine lactoferrampin (LFampB) is an antimicrobial peptide identified through sequence analysis of bovine lactoferrin [24] with broad range antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria as well as candidacidal activity [25–28]. Freeze-fracture transmission electron microscopy of *E. coli* and *C. albicans* cells treated with LFampB revealed that the peptide causes partial disintegration of the plasma membrane [29], suggesting that the primary target of lactoferrampin is the cytoplasmic membrane.

The solution structures and membrane interactions of bovine and human lactoferrampins have been extensively characterized [30–32]. Like many antimicrobial peptides, lactoferrampin is unstructured in aqueous solution and it only adopts a helical conformation in the presence of lipids. The sodium dodecyl sulphate (SDS) micelle-bound NMR solution structure of lactoferrampin revealed an amphipathic helix in the N-terminal region of the peptide, while the cationic C-terminal region is devoid of regular secondary structure. Interestingly, removal of the positive charges in the C-terminal region decreases the antimicrobial potency of the peptide [26, 28] and adding positive charges to this region increases the antimicrobial activity [32]. Based on these results, a model of antimicrobial activity has been described wherein the cationic C-terminal region mediates the initial attraction of lactoferrampin to the negatively charged bacterial surface. This electrostatic attraction brings lactoferrampin into close proximity to the bacterial membrane, allowing for folding and insertion of the N-terminal helix into the bilayer, ultimately leading to membrane perturbation and cell death.

In the present work, the antimicrobial mechanism of action of a unique hybrid antimicrobial peptide composed of LFampB and LFcin17–30 is examined. The heterodimeric peptide (named LFchimera) is coupled together through the two amino groups of a single lysine residue at the C-terminal ends of both constituent peptides. In addition, the carboxyl group on the lysine linker is amidated to remove the negative charge (Fig. 1B). The rationale behind the design of the LFchimera was to link these two peptides in a manner that could maintain their relative orientation as seen in the crystal structure of bovine lactoferrin [18]. Since many of the biological host-defence activities of lactoferrin are localized in the cationic N-terminal lobe of the protein, it was hypothesized that joining LFampB to LFcin through this Lys linker might maintain the spatial orientation of these two sequences and enhance the activity of this heterodimer. Indeed, extensive characterization of the activity of LFchimera has shown it to be remarkably more potent against Gram-positive and Gram-negative bacteria compared to either of the constituent peptides [33, 34]. Additionally, the LFchimera has been found to possess antiparasitic activity [35] and even antibiotic resistant strains of *Staphylococcus aureus* and *Escherichia coli* are susceptible to this hybrid peptide [36]. Together, these studies highlight the potential of LFchimera as a future treatment option for serious bacterial infections. However, the structure of LFchimera and the molecular mechanisms underlying the enhanced antimicrobial activity remain to be elucidated.

In the present work, the effect of the peptides on the thermotropic phase behaviour of anionic phospholipids was examined using differential scanning calorimetry (DSC) [37]. Furthermore, the membrane perturbing properties of LFchimera and its constituent peptides was evaluated by monitoring the release of the self quenching dye, calcein, from large unilamellar vesicles (LUVs) of varying lipid composition. Peptide induced liposome fusion was also assessed as this is a feature that has previously been reported for the full length bovine LFcin peptide [38]. Structural characterization of all the peptides was performed using circular dichroism (CD) spectroscopy and high resolution nuclear magnetic resonance (NMR) spectroscopy. The results presented here reveal that the LFchimera has a stronger membrane perturbing effect than either LFampB or LFcin17–30 or a mixture of these two peptides. The CD and NMR structural results demonstrate that the LFchimera has increased helical content compared to the two constituent peptides, suggesting that the Lys linkage influences the structure of the LFampB and LFcin17–30 regions of LFchimera. The outcome of these experiments is discussed with respect to the previously reported antimicrobial activity of LFampB, LFcin17–30 and LFchimera. In addition, the merits of using a co-solvent mixture as a mimic for biological membranes in NMR spectroscopy are also considered.

2. Methods and materials

2.1. Peptide synthesis

Synthesis of LFcin17–30 and LFampB was performed in the same manner as described previously [29, 32]. The synthesis of LFchimera