Variation in antimicrobial activity of lactoferricin-derived peptides explained by structure modelling

Sebastien Farnaud a,*, Alpesh Patel a, Edward W. Odell b, Robert W. Evans a

a Randall Division of Cell and Molecular Biophysics, King’s College London, New Hunt’s House, Guy’s Campus, St. Thomas Street, London SE1 1UL, UK
b Division of Oral Pathology, King’s College London, GKT Dental Institute, London, UK

Received 12 July 2004; accepted 17 July 2004
First published online 28 July 2004

Abstract

Antimicrobial peptides bovine lactoferricin (LfcinB) and human lactoferricin (LfcinH) are produced from the respective lactoferrin, but are more active than their precursors. Despite sequence homology, the bovine peptide and its derivatives are more active than their human homologs. Such differences between not only the peptides and their precursor but also between the bovine and the human peptides could relate to structural differences. Upon sequence alignment of both peptides with their parental proteins, the structural differences observed between the bovine lactoferrin (BLf) and LfcinB were also found between the human lactoferrin (HLf) and the LfcinH. The helical structures in HLF are replaced by β-strands separated by a strong turn in LfcinH suggesting an antiparallel β-sheet structure similar to LfcinB. MIC assays with HLP-2 and BLP-2, 11-residue peptides derived from the active core of both Lfcins, against Escherichia coli, showed that the bovine derivative, BLP-2, is more active than its human homolog HLP-2. Both 3D models for HLP-2 and BLP-2 showed that the β-strand is centred between the aromatic residues giving both side chains the same orientations. The displacement towards the N-terminus observed for the β-strand in HLP-2, compared with its central location in BLP-2, could be less favourable to membrane interaction and therefore responsible for the decrease in activity. Such a model suggests for LfcinH a mechanism similar to the one observed for LfcinB, where the absence of long-range interaction, present in lactoferrin, destabilises the first alpha helix, as observed in solution and, upon interaction with the membrane, could result in the formation of a β-strand, as observed in the presence of LPS. The location of the β-strand in relation to the positive charges, seems to define the efficiency of the activity of the peptide and may explain the difference in activity obtained between HLP-2 and BLP-2.

Keywords: Lactoferrin; Lactoferricin; Cationic antimicrobial peptides; LPS; Modeller

1. Introduction

The increasing number of antimicrobial cationic peptides identified from a wide range of organisms indicates an important contribution in the immune response [1]. It also suggests an interesting model for the design of new molecules in the ever-increasing fight against infectious agents. Under the name of cationic peptides are grouped positively charged naturally occurring antimicrobial peptides that display a great variety of primary, secondary and tertiary structure. Such diversity explains why no consensus mode of action has been described for the whole group. Amongst their different physicochemical properties, their cationicity, hydrophobicity and...
secondary structure content have been shown to be the main factors implicated in their antimicrobial effect. In addition, their wide range of targets which comprises not only Gram-negative and -positive bacteria but also viruses, parasites and malignant cells, also suggests different modes of action [2]. Studies on antimicrobial activity against Gram-negative bacteria indicate that the membrane is the main site of action, although further interactions inside the cytoplasm have also been suggested [3].

Human and bovine lactoferricins (LfcinH, LfcinB) are cationic peptides which are released upon proteolytic cleavage of the N-terminus of intact human or bovine lactoferrins (HLf, BLf), respectively [4]. Lactoferrins (Lfs) are members of the transferrin family of iron proteins, which are present in milk and other secretions including saliva, tears, seminal fluids, mucins and the secondary granules of neutrophils [5]. The protein is found not only in humans but also in a large number of mammals with a highly conserved sequence. HLF and BLf have been shown to have, amongst numerous properties, some antimicrobial activity. However, in both cases, their N-terminus derived peptides, LfcinH and LfcinB, have been found to be more active than their respective precursors, which suggests a different mechanism. The X-ray structures of lactoferrins have been determined from several organisms [6,7] and they all show a high degree of similarity in secondary and tertiary structure. However, only the structure of LfcinB structure has been solved [8]. By comparing the tertiary structure of LfcinB and its corresponding fragment within the native lactoferrin, a difference in conformation was observed between the two identical primary structures. Such an α-β polymorphism has been observed for a number of proteins, including the prion structures. Such an α-β polymorphism has been observed for a number of proteins, including the prion structures. Such an α-β polymorphism has been observed for a number of proteins, including the prion structures. Such an α-β polymorphism has been observed for a number of proteins, including the prion structures.

In previous work [11,12] with HLP-2, a synthetic 11-residues peptide corresponding to the region (21–35) of the LfcinH equivalent to the surface region in HLF. In a circular dichroism (CD) study of the interaction of HLP-2 with E. coli LPS, Chapple et al. [13] have just shown that conformational changes were associated with binding. The peptides were shown to adopt a β-strand rather than an α-helical conformation which resulted in an ordered formation of the aggregates.

In the present study, the structure of the LfcinH-derived peptide HLP-2 was investigated. Based on the solved 3D structure of the LfcinB, a model was built for HLP-2. The results were compared with the structure of the corresponding sequence in the native lactoferrin and structural differences were related to differences in antimicrobial activity.

2. Material and methods

2.1. Peptides and bacterial strains

HLP-2 and BLP-2 peptides (BLP-2 NH2-RRWQWR MKKLG-COOH, HLP-2 NH2-FQWQRN MRKVR-COOH) Peptides were synthesised by Advanced Biotechnology Centre (ABC) (Imperial College London) using an automatic synthesiser and analysed by HPLC. They were stored in their freeze-dried form at −20 °C and dissolved on the day of use, as described in the method of Hancock (http://www.cmdr.ubc.ca/bobh). The peptides were assayed against E. coli wild type strain W3110 obtained from the CGSC, E. coli Genetic Stock Center.

2.2. Minimal inhibitory concentration and minimal bacteriocidal concentration measurement

Minimal inhibitory concentrations (MICs) and minimal bacteriocidal concentrations (MBCs) (Table 1) were obtained using the modified method used in the Hancock laboratory (http://www.cmdr.ubc.ca/bobh/) which is based on the classical microtitre broth dilution recommended by the National Committee of Laboratory Safety and Standards (NCLSS).

2.3. Secondary structure prediction, sequence alignment and structure modelling

Sequence and structure of the bovin lactoferricin (LfcinB) (1LFC.pdb), bovin lactoferrin (BLf)
(1BLF.pdb), buffalo lactoferrin (BoLf) (1CE2.pdb), horse lactoferrin (HoLf) (1B1X.pdb), camel lactoferrin (CLf) (1H6Q.pdb), goat lactoferrin (GLf) (1JW1.pdb) and human lactoferrin (HLf) (1SQY.pdb) were obtained from the Protein Data Base (PDB). Alignments of amino acid sequences were obtained using the program Clustalw available from the Expasy server. The final graphic display was obtained using the software Bioedit. Secondary structure predictions were obtained by using programmes the program PeptideStructure from the Wisconsin Package Version 10.3. Secondary structure prediction was only carried out for the region of LfcinH (Val12-Ile47), the equivalent of LfcinB. 3D models were obtained from both peptides BLP-2 and HLP-2 based on the solved structure of LfcinB (1LFC.pdb), using Modeller 6v2 on a Silicon Graphic System and the final models were chosen after energy minimisation.

3. Results

HLf sequence was aligned with BLf sequence and the corresponding part of the sequence of all other available solved lactoferrin structures, namely buffalo, goat, horse and camel lactoferrins. The secondary structures of the solved structures were also aligned and compared with the predicted secondary structure of the LfcinH (Fig. 1). The program PeptideStructure from the GCG Wisconsin package was used to predict the secondary structure of LfcinH. The Chou–Fasman [14] method used in this program is well adapted for the prediction of a peptide structure since it does not relate directly to the sequence alignment with known 3D structure, which in this case would only result in the structure of the corresponding part of the N-terminus of HLf structure. This method is a statistical prediction method based on the calculation of the statistical propensities of each residue to form either an \( \alpha \)-helix or a \( \beta \)-strand. This is the method of choice for short peptides where little interaction is expected with distant residues. Since the structural comparison was limited to the bovine peptide, the prediction for LfcinH was only done over the region (Val12-Ile47) homologous to the entire LfcinB. The predicted secondary structure obtained for the region of LfcinH was different from the corresponding part for the sequence in HLf. The first \( \alpha \)-helix located between Q14 and N26 in HLf has been replaced by a shorter \( \beta \)-strand extending from A17 to W23. Similarly, the C-terminus of the peptide, which in HLf is part of the second \( \alpha \)-helix, corresponds in the predicted structure to a \( \beta \)-strand. The turn located between both \( \beta \)-strands would suggest a \( \beta \)-sheet structure, which would be similar to the structure of LfcinB. The primary structures of LfcinB and LfcinH share 36% identity and 52% similarity and though their secondary structures are not superimposable, some strong homology can be identified in their composition and distribution, with both structures comprising two \( \beta \)-strands separated by a turn. The alignment over the HLP-2 region, the active core of the peptide, with BLP-2, its counterpart in the LfcinB (Fig. 2), shows the same secondary structure content with a shift of the \( \beta \)-strand towards the N-terminus. After determining the MIC for both peptides (Table 1), BLP-2 was found to be more active than HLP-2. Such a result was expected from previous results with LfcinH and derivate peptides which always indicates the bovine counterpart to be more active.

In an attempt to relate the difference observed in activity with the structural shift in secondary structure, a model of HLP-2 was built using the program Modeller, using LfcinB as a template (Fig. 3). The 3D model of both peptides shows that both \( \beta \)-strands, though not at the same location in both peptides, orientate the side chains of both aromatic residues on the same side of the peptide. Such an orientation would facilitate interaction with the lipidA in the second step of the interaction with the outer membrane. The main difference between the peptides is in the relative distribution

![Fig. 1. Multiple alignment of the sequences of lactoferrins (Lfs) and lactoferricins (Lfcins) and their secondary structures. Identities are highlighted in black and similarities in grey; secondary structure alignment: H = helix; E = extended beta strand; T = hydrogen bonded turn; S = bend.](https://academic.oup.com/femsle/article-abstract/238/1/221/546938)
of their positive charges; in BLP-2, positive charges are located on both sides of the β-strand whereas in HLP-2, the positive charges are only located on the C-terminus of the peptide.

4. Discussion

LfcinB and LfcinH are antimicrobial peptides derived from the N-terminal region of BLf and HLf, with greater activity than their respective precursors. Interaction of the peptides with the membrane of microorganisms is thought to be central to their mode of action. Though strong sequence similarity between both peptides suggests structural homology, the bovine peptide has a stronger antimicrobial activity than the human peptide. The corresponding sequence in the full length proteins has also been found to be the site of interaction with LPS of Gram negative bacteria [15,16]. The structure of the LfcinB has been solved by NMR [8] and was found to be different from the X-ray structure of the same sequence in the intact BLf. In the X-ray crystal structure, an alpha helix extends from Gln13 to Met26, overlapping the region from Phe1 to Met10 in LfcinB, and a β-strand extending from Ile34 to Arg38 (Ile18 to Arg22 in LfcinB) participates in a parallel β-sheet with another N-terminal β-strand of the native lactoferrin. The 14-residue helix is not present in the solution structure of LfcinB but is replaced by an extended structure which forms a β-strand. Such a structural difference implies different dynamics for interaction with the membrane which might account, in part, for the difference in activity observed between the peptide and its precursor. To investigate the mode of action of LfcinH against Gram-negative bacteria, the interaction of derivative peptides HLP-2 and HLP-6, corresponding to the active core, with E. coli LPS have been studied [13]. Upon binding to the membrane, both peptides were found to adopt a β-strand conformation, rather than the α-helical conformation expected from the HLf structure. In addition, a time-dependent association of peptides was observed resulting in an ordered formation of peptide aggregates.

The aim of this work was to investigate whether the difference in antimicrobial activity between the bovine and the human peptides could be explained by structural differences between the peptides. Since the 3D structure of HLfcin has not been solved, its secondary structure can only be predicted. In contrast to the structure observed in the N-terminus of HLf, the prediction did not show any α-helical structure in LfcinH but instead, two β-strands separated by a strong turn. Such a difference between the peptide and its precursor is reminiscent of the difference observed between LfcinB and BLf. This suggests that LfcinH might adopt an antiparallel β-sheet structure similar to the LfcinB, which contrasts with the α-helical content of the equivalent region in the intact human and BLf. The native lactoferrin structure is the result of neighbour residue interactions but also long range hydrophobic interactions where some of the N-terminal residues are buried in domain 1 and interact with residues from domain 2 [17]. In the absence of long-range interactions observed in lactoferricin, the substitution of helical structures by β-strands stabilises the whole structure by forming a β-sheet that orients the functionally important residues. In LfcinB, the presence of an extended hydrophobic surface with hydrophilic and positively charged residues surrounding the hydrophobic surface results in an amphipathic character. The conservation of many of these residues in LfcinH implies that a similar property can be expected for the human peptide.

In an attempt to understand differences in activity between the bovine and human peptides, MIC assays and structure modelling were carried out using two
peptides HLP-2 and BLP-2 derived from the active core of both peptides. As expected from the results from the previous studies with Lfcins, the bovine derivative BLP-2 was found to be more active than the human peptide HLP-2. In order to understand such a difference, 3D models based on LfcinB structure were built for HLP-2 and BLP-2. In both models, the β-strand is centred between the aromatic residues and gives both side chains the same orientation. For membrane interaction, such an orientation seems favourable for residues expected to interact with the hydrophobic core of the membrane. The displacement of the β-strand towards the N-terminus observed in the HLP-2, corresponds to the displacement of the hydrophobic core of the peptide. In BLP-2, the β-strand is located between positives charges whereas in HLP-2, the charges are mainly located at the opposite end of the peptide. The importance of the relative positions of aromatic and positively charged residues has been shown in a previous model for the of interaction peptides with gram-negative bacteria [10]. In this two-step mechanism, positive residues of the cationic peptide are proposed to first interact with negative charges carried by the LPS in order to disorganise the structure of the outer membrane. Such an interaction permits the second step where the tryptophan residues approach the lipid-A for hydrophobic interactions, leading to further penetration of the outer membrane. The distribution of charges surrounding the hydrophobic core in BLP-2 might be more favourable to this mechanism than the one observed in HLP-2. From these results, it appeared that the absence of long-range interactions observed in lactoferrin destabilise the first alpha helix in BLP-2 and HLP-2 resulting in random conformation in solution. In the presence of LPS a β-strand structure seems to be better suited for hydrophobic interaction. In addition, in order to understand the mechanism of peptide/membrane interaction, peptide/peptide interaction should also be taken in consideration. The recently described ordered association of peptides upon interaction with the membrane implies that the distribution of particular residues within the peptides is also an important factor for peptide association. The conformational changes, which correspond to the transition from a random to an organised state observed upon addition of LPS to the peptides in solution [13], seem to be triggered by the primary interaction of LPS with the peptides, which adopt a β conformation that is more favourable to the interaction. Such a change in peptide conformation might initiate further conformational changes in other peptides promoting, in turn, peptide/peptide interaction. Such a mechanism could be similar for both HLP-2 and BLP-2 peptides, but the more balanced distribution of charges observed in BLP-2 might lead to more efficient peptide/membrane and peptide/peptide associations.

Acknowledgement

Sebastien Farnaud was funded by a Wellcome Trust Project Grant (Reference No. 059414) and Alpesh Patel was funded by a studentship from the BBSRC.

References

[1] Hancock, R.E. and Lehrer, R. (1998) Cationic peptides: a new source of antibiotics. Trends Biotechnol. 16, 82–88.
[2] Martin, E., Ganz, T. and Lehrer, R.I. (1995) Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukoc. Biol. 58, 128–136.
[3] Ulvatne, H., Haukland, H.H., Olsvik, O. and Vorland, L.H. (2001) Lactoferricin B causes depolarization of the cytoplasmic membrane of Escherichia coli ATCC 25922 and fusion of negatively charged liposomes. FEBS Lett. 492, 62–65.
[4] Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. and Tomita, M. (1992) Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta 1121, 130–136.
[5] Farnaud, S. and Evans, R.W. (2003) Lactoferrin – a multifunctional protein with antimicrobial properties. Mol. Immunol. 40, 395–405.
[6] Baker, E.N., Anderson, B.F., Baker, H.M., MacGillivray, R.T., Moore, S.A., Peterson, N.A., Shewry, S.C. and Tweetide, J.W. (1998) Three-dimensional structure of lactoferrin. Implications for function, including comparisons with transferrin. Adv. Exp. Med. Biol. 443, 1–14.
[7] Baker, E.N., Baker, H.M. and Kidd, R.D. (2002) Lactoferrin and transferrin: functional variations on a common structural framework. Biochem. Cell Biol. 80, 27–34.
[8] Hwang, P.M., Zhou, N., Shan, X., Arrowsmith, C.H. and Vogel, H.J. (1998) Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin. Biochemistry 37, 4288–4298.
[9] Mihara, H. and Takahashi, Y. (1997) Engineering peptides and proteins that undergo alpha-to-beta transitions. Curr. Opin. Struct. Biol. 7, 501–508.
[10] Farnaud, S., Spiller, C., Moriarty, L.C., Patell, A., Gant, V., Odell, E.W. and Evans, R.W. (2004) Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity. FEMS Microbiol. Lett. 233, 193–199.
[11] Odell, E.W., Sarra, R., Foxworthy, M., Chapple, D.S. and Evans, R.W. (1996) Antibacterial activity of peptides homologous to a loop region in human lactoferrin. FEBS Lett. 382, 175–178.
[12] Chapple, D.S., Mason, D.J., Joannou, C.L., Odell, E.W., Gant, V. and Evans, R.W. (1998) Three-dimensional solution structure of lactoferricin B. Antimicrob. Agents Chemother. 42, 2190–2198.
[13] Chapple, D.S., Hussain, R., Joannou, C.L., Hancock, R.E., Odell, E., Evans, R.W. and Siligardi, G. (2004) Structure and association of human lactoferrin peptides with Escherichia coli lipopolysaccharide. Antimicrob. Agents Chemother. 48, 2190–2198.
[14] Chou, P.Y. and Fasman, G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45–148.
[15] Elass-Rochard, E., Roseanu, A., Legrand, D., Trif, M., Salmon, V., Motas, C., Montreuil, J. and Spik, G. (1995) Lactoferrin-lipopolysaccharide interaction: involvement of the 28–34 loop region of human lactoferrin in the high-affinity binding to Escherichia coli i05BS lipopolysaccharide. Biochem. J. 312 (Pt 3), 839–845.
[16] Caccavo, D., Pellegrino, N.M., Altamura, M., Rigon, A., Amati, L., Amoroso, A. and Jirillo, E. (2002) Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application. J. Endotoxin Res. 8, 403–417.

[17] Sun, X.L., Baker, H.M., Shewry, S.C., Jameson, G.B. and Baker, E.N. (1999) Structure of recombinant human lactoferrin expressed in Aspergillus awamori. Acta Crystallogr. D 55 (Pt. 2), 403–407.