Journal of Peptide Science
J. Peptide Sci. **7**: 190–196 (2001)
DOI: 10.1002/psc.318

The Role of Tryptophan in the Antibacterial Activity of a 15-Residue Bovine Lactoferricin Peptide

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Received 4 December 2000
Accepted 2 January 2001

Abstract: Bovine lactoferricin is a 25-residue antibacterial peptide isolated after gastric cleavage of the iron transporting protein lactoferrin. A 15-residue fragment, FKCRRWQWRMKKLGA of this peptide sustains most of the antibacterial activity. In this truncated sequence, the two Trp residues are found to be essential for antibacterial activity. The anchoring properties of Trp, as have been observed in membrane proteins, are believed to be important for the interaction of Trp containing antibacterial peptides with bacterial cell membranes. We have investigated the molecular properties which make Trp important for the antibacterial activity of the 15-residue peptide by replacing Trp with natural and unnatural aromatic amino acids. This series of peptides was tested for antibacterial activity against *Echerichia coli* and *Staphylococcus aureus*. We found that neither the hydrogen bonding ability nor the amphipathicity of the indole system are essential properties for the effect of Trp on the antibacterial activity of the peptides. Replacement of Trp with residues containing aromatic hydrocarbon side chains gave the most active peptides. We propose that aromatic hydrocarbon residues are able to position themselves deeper into the bacterial cell membrane, making the peptide more efficient in disrupting the bacterial cell membrane. From our results the size, shape and aromatic character of Trp seem to be the most important features for the activity of this class of Trp containing antibacterial peptides. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial peptide; lactoferricin; tryptophan; unnatural aromatic amino acids

INTRODUCTION

Antibacterial peptides constitute a novel class of antibiotics that is getting more and more attention (for recent reviews see [1,2]). Structural diversity among antibacterial peptides is large, but the peptides are commonly highly cationic, with a propensity to form amphipathic secondary structures on interaction with negatively charged bacterial cell membranes. The mechanism of action is not clearly understood for all of these peptides, and several models to explain their actions have been put forward [1–4]. The peptides are believed to exert their antibacterial activity by disrupting the bacterial cell membrane, either by forming pores or by a general weakening of the membrane (carpet mechanism). Intracellular structures are also proposed to be targets for the peptides [2].

Bovine lactoferricin is a 25-residue antibacterial peptide isolated after gastric cleavage of the bovine iron chelating protein lactoferrin [5]. The peptide consists of residues 17–41 of the lactoferrin protein. A 15-residue peptide, FKCRRWQWRMKKLGA (LFB), comprising only the α-helical 17–41 sequence, has been shown to sustain most of the antibacterial activity [6]. Recently, our research group has shown that the two Trp residues

Abbreviations: ATCC, American Type Culture Collection; Bal, β-(benzothien-3-yl)alanine; LFB, lactoferricin B residues 17-31; MIC, minimal inhibitory concentration; 1-Nal, β-(naphth-1-yl)alanine; 2-Nal, β-(naphth-2-yl)alanine; PAC-PEG-PS, 4-hydroxy methylphenoxy acetic acid-polyethylene glycol-polystyrene resin; SDS, sodium dodecyl sulfate.

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are essential for the antibacterial activity of LFB, and that the introduction of one extra Trp in human, goat and porcine lactoferricins increases the antibacterial activity of these peptides up to six-fold [7]. The importance of Trp in other antibacterial peptides has recently been shown by Bikshapathy *et al.* [8] where the introduction of one and two Trp residues in the antibacterial peptide PKLLTKFLKSWIG resulted in increased antibacterial and haemolytic activity. Introduction of Trp in cyclic bactenecin, a cationic antimicrobial peptide from bovine neutrophils, also resulted in a substantial increase of antibacterial activity [9]. Several peptides with a high content of Trp, such as tritrpticin [10] and indolicidine [11], have been characterized and investigated for antimicrobial activity. The high content of Trp in these peptides and the importance of Trp for the antibacterial activity of LFB suggest that Trp plays a crucial role in the interaction with bacteria.

The membrane-disrupting properties of cecropin A(1–8)-magainin 2 (1–12) hybrid peptides have been suggested to be dependent on both the amphipathicity and the hydrophobicity of the peptides [12]. A blue shift in Trp fluorescence emission spectra has been observed upon interaction of the hybrid peptides with phospholipid vesicles. This indicates that the Trp residue has been moved into the nonpolar environment of the lipid vesicle. This effect of Trp has also been established for several other peptides, such as gramicidin [13], where Trp is essential for the pore-forming properties of the peptide. Aromatic residues have been found to occur preferentially at the cytoplasmic boundary in transmembrane segments of membrane proteins, with Trp also favoured at the extracellular boundary [14]. Trp residues are suggested to serve as membrane 'anchors' for membrane proteins [15,16]. Wimley and White [17] have shown that among the naturally occurring amino acids, the three aromatic amino acids Trp, Tyr and Phe favour the partition of Ac-Trp-Leu-Xxx-Leu-Leu pentapeptides into the membrane interface.

Trp has been pointed out to be an especially important residue in antibacterial peptides because the indole nucleus has both lipophilic and polar properties. For membrane proteins, the indole is suggested to be inserted into the membrane, with the hydrophobic part interacting with the hydrophobic portion of the bilayer, and the amine function interacting more closely with the polar head-groups in the proximity of the outside of the membrane [15]. On the other hand, Yau *et al.* have recently suggested that it is the aromaticity of Trp that gives rise to the membrane anchoring properties [18].

In this study, we will address which molecular properties makes Trp essential for the activity of the LFB peptides. In order to accomplish this, peptides where Trp was replaced by natural and unnatural aromatic amino acids with different properties were synthesized and tested for antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were synthesized on a Millipore 9050 Plus PepSyntheziser (Milligen, Milford, MA, USA) using Fmoc protection, and activation with Pfp-esters or *in situ* activation using HATU or HBTU as coupling reagents in DMF. HOBt (1.3 eq.) was added to catalyse coupling reactions with Pfp-esters. When using HATU or HBTU (1 eq.), reactions were base-catalysed with DIPEA (2.4 eq.). A four-fold excess of amino acids was employed during every coupling step. For unnatural amino acids in some cases a two-fold excess was employed. The *C*-terminal amino acid (alanine in all peptides) was preattached to a 4-hydroxymethylphenoxy acetic acid-polyethylene glycol-polystyrene (PAC-PEG-PS) resin, which ensures a free *C*-terminal carboxylic acid after final acid treatment (see below). This resin was used for all peptide syntheses. All amino acids with reactive side chains, except cysteine, were protected with acid-labile protecting groups and deprotected during cleavage of the peptide from the solid support upon treatment with Reagent K [19] (82.5% TFA, 5% thioanisole, 2.5% ethanedithiol and 5% water) for no more than 3 h. Cysteine was irreversibly protected in all peptides with an Acm group. After cleavage, the solid support was removed by filtration, and the filtrate concentrated under reduced pressure. The crude peptides were precipitated from diethyl ether. After washing the precipitates several times with diethyl ether, the crude peptides were dried under reduced pressure.

Chemicals

All natural Fmoc-amino acids, Fmoc-L-Ala-PEG-PS resin, 1-HOBt, HATU, HBTU, DMF, piperidine, DIPEA and TFA were purchased from PerSeptive Biosystems (Hertford, UK). Fmoc-β-(benzothien-3-yl)alanine (Fmoc-Bal), Fmoc-β-(naphth-1-yl)alanine

(Fmoc-1-Nal) and Fmoc-β-(naphth-2-yl)alanine (F-moc-2-Nal) were purchased from Bachem (Bubendorf, Switzerland).

Purification and Analysis of the Peptides

The peptides were purified by preparative reverse-phased high performance liquid chromatography (RP-HPLC) using a C_{18}-column (Delta-Pak™ C18, 100 Å, 15 μm, 25 × 100 mm, Waters Corp., Milford, MA, USA) with a mixture of water and acetonitrile (both containing 0.1% TFA) as mobile phase and UV-detection at 254 nm or 214 nm where necessary (LFB Phe 6,8). The homogeneity of the purified peptides was analysed on an analytical C_{18} HPLC column (Delta-Pak™ C18, 100 Å, 5 μm, 3.9 × 150 mm, Waters Corp.). The purity of all peptides was found to be above 95%. The integrity of the peptides was checked by positive ion electrospray ionization mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, UK).

Antibacterial Activity

The bacterial strains, *E. coli* American Type Culture Collection (ATCC) 25922 and *S. aureus* ATCC 25923, were grown in 2% Bacto Peptone water (Difco 1807-17-4) until exponential growth. A standard microdilution technique with an inoculum of 2×10^6 colony-forming units per mL was used. The minimal inhibitory concentration (MIC) of the peptides was determined in 2% Bacto Peptone water after incubation overnight at 37°C. The concentration range used for the modified LFB peptides was between 300 and 1.0 μg/mL. All peptides were tested in duplicates.

RESULTS

To probe the importance of different molecular properties of Trp, a series of peptides was prepared where residues Trp 6, Trp 8 and both Trp 6 and Trp 8 of LFB were replaced with different aromatic amino acids. The unnatural amino acids used as replacements for Trp in this study are given in Figure 1. The antibacterial activities for LFB and the modified analogues against *E. coli* and *S. aureus* are compiled in Table 1. Incorporation of different aromatic amino acids revealed that the antibacterial activity and bacterial selectivity of the LFB derivatives are highly dependent on the structural parameters of the aromatic residues.

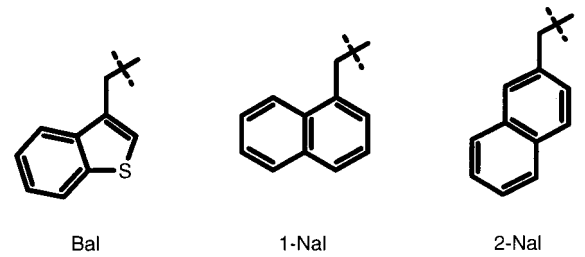

Figure 1 Structure of side chains and abbreviations for the Trp analogues employed.

The replacement of Trp with Bal gave an increase in activity against both bacterial strains, with MIC-values in the range of 15–20 μg/mL against *E. coli* and 25–75 μg/mL against *S. aureus*, indicating that the indole nitrogen is not essential.

The two naphthylalanine isomers gave different effects when incorporated in the LFB sequence. Of the peptides containing unnatural amino acids, LFB 1-Nal6 is the only one in this study that gave a decrease in antibacterial activity against *S. aureus*, with a MIC-value of 150 μg/mL. Against *E. coli*, the activity of LFB 1-Nal6 was equal to that of the original LFB. Replacement of Trp in position 8 with 1-Nal gave a peptide equally active to LFB against *S. aureus*, but showed a 2.5-fold increase in activity against *E. coli*. Replacement of both Trp residues with 1-Nal gave a peptide with increased activity against both bacterial strains, with MIC-values of 10 and 50 μg/mL against *E. coli* and *S. aureus*, respectively. Incorporation of 2-Nal in the LFB sequence gave peptides with MIC-values against *E. coli* of 20 μg/mL for LFB 2-Nal6 and 10 μg/mL for LFB 2-Nal8 and LFB 2-Nal6,8. The 2-Nal peptides were considerably more active against *S. aureus* than the 1-Nal derivatives, with MIC-values of 75, 50 and 20 μg/mL for LFB 2-Nal6, 2-Nal8 and 2-Nal6,8, respectively.

A severe loss of antibacterial activity against *S. aureus* was observed when Phe replaced either of the Trp residues. These peptides showed MIC-values above 300 μg/mL against *S. aureus*. The antibacterial activity against *E. coli* ranged from 25 to 150 μg/mL, with LFB Phe6,8 being the poorer derivative.

DISCUSSION

The indole side chain of Trp has molecular properties that are important both for the anchoring of

Table 1 Antibacterial Activity of LFB Derivatives

Peptide name	Molecular mass[a]		Sequence															MIC[b] *E. coli*		MIC[b] *S. aureus*	
LFB	2064.8	(2064.5)	F	K	C	R	R	W	Q	W	R	M	K	K	L	G	A	50	(24)	100	(48)
LFB Bal6	2082.6	(2082.0)	–	–	–	–	–	Bal	–	W	–	–	–	–	–	–	–	15	(7.2)	75	(36)
LFB Bal8	2082.6	(2081.8)	–	–	–	–	–	W	–	Bal	–	–	–	–	–	–	–	15	(7.2)	35	(17)
LFB Bal6,8	2099.7	(2098.4)	–	–	–	–	–	Bal	–	Bal	–	–	–	–	–	–	–	20	(9.5)	25	(12)
LFB 1-Nal6	2076.6	(2076.8)	–	–	–	–	–	1-Nal	–	W	–	–	–	–	–	–	–	50	(24)	150	(72)
LFB 1-Nal8	2076.6	(2077.0)	–	–	–	–	–	W	–	1-Nal	–	–	–	–	–	–	–	20	(9.6)	100	(48)
LFB 1-Nal6,8	2087.6	(2086.9)	–	–	–	–	–	1-Nal	–	1-Nal	–	–	–	–	–	–	–	10	(4.8)	50	(24)
LFB 2-Nal6	2076.5	(2075.8)	–	–	–	–	–	2-Nal	–	W	–	–	–	–	–	–	–	20	(9.6)	75	(36)
LFB 2-Nal8	2076.5	(2076.0)	–	–	–	–	–	W	–	2-Nal	–	–	–	–	–	–	–	10	(4.8)	50	(24)
LFB 2-Nal6,8	2087.5	(2087.0)	–	–	–	–	–	2-Nal	–	2-Nal	–	–	–	–	–	–	–	10	(4.8)	20	(9.6)
LFB Phe6	2026.5	(2025.8)	–	–	–	–	–	F	–	W	–	–	–	–	–	–	–	50	(25)	>300	(>148)
LFB Phe8	2026.5	(2025.8)	–	–	–	–	–	W	–	F	–	–	–	–	–	–	–	25	(12)	>300	(>148)
LFB Phe6,8	1987.5	(1986.0)	–	–	–	–	–	F	–	F	–	–	–	–	–	–	–	150	(76)	>300	(>151)

[a] Calculated and (observed) molecular mass including Acm-protected cysteine.
[b] Minimal inhibitory concentration in μg/mL and (μM).

proteins in membranes, and for the antibacterial activity of LFB peptides. In this work, we have studied the importance of three different molecular properties of the Trp side chain, namely the size, amphipathicity and the ability to participate in hydrogen bonding. To gain insight into whether the hydrogen bonding ability of the indole amine function is a key structural element of the effect of Trp on the antibacterial activity of LFB, a series of peptides where Trp was replaced by Bal were synthesized. The benzothienyl side chain of Bal is a sulfur analogue of the indole side chain of Trp. As opposed to the indole amine, the sulfur atom does not have the ability to participate in hydrogen bonding. When tested against bacteria, the Bal peptides showed an increase in antibacterial activity against both *S. aureus* and *E. coli*. Replacement of nitrogen with a sulfur atom in the side chain of Trp resulted in an up to 3.3-fold increase in activity against *E. coli* and an up to four-fold increase in activity against *S. aureus*. These results indicate that the hydrogen bonding ability of the indole is not essential for the effect of Trp on the activity of LFB peptides. Having established that hydrogen bonding was not essential, the importance of the amphipathic nature of Trp was investigated. The results from the Bal peptides suggest that the amphipathicity of Trp was not essential either, as the benzothienyl moiety of Bal can not be considered to be very amphipathic, even though it contains a heteroatom. The low importance of amphipathicity in the Trp side chain was further substantiated by replacement of Trp by non-amphipathic aromatic residues in LFB derivatives. 1-Nal and 2-Nal (Figure 1) are aromatic amino acids of comparable size to Trp, that can not be considered to be amphipathic as Trp, and to a smaller extent Bal, since the naphthalene side chains do not contain any heteroatoms. Replacing the Trp residues with 1-Nal and 2-Nal gave peptides that had a more diverse ability to inhibit bacterial growth than the Bal peptides. The 2-Nal peptides showed MIC-values in the range of 10–20 μg/mL against *E. coli* and 20–75 μg/mL against *S. aureus*. The unmodified LFB shows MIC-values of 50 μg/mL and 100 μg/mL against *E. coli* and *S. aureus*, respectively. LFB 2-Nal6,8 was the most active derivative against both bacterial strains. The 1-Nal peptides were somewhat less active than the 2-Nal isomers; LFB 1-Nal6 and LFB 1-Nal8 are the only derivatives containing unnatural amino acids in this study where the antibacterial effect is not increased against *S. aureus*. Only when both Trp residues are replaced by 1-Nal, is the antibacterial effect increased compared to LFB, two-fold against *S. aureus* and five-fold against *E. coli*. The results from the Nal peptides clearly demonstrate that the hydrophobic aromatic side chains are favourable for the interaction between the peptides and the bacterial cell membranes. The difference between 1-Nal and 2-Nal peptides showed that the shape of the aromatic amino acid side chain influences the properties of the peptides to a considerable extent.

The substitution pattern of the naphthalene ring is the only structural difference between 1-Nal and 2-Nal, and it obviously has an impact on the ability of the peptides to kill bacteria. As can be seen from Figure 1, the naphthalene moiety is pointing more away from the β-carbon atom in 2-Nal the than it is in 1-Nal. The difference in the substitution pattern of the aromatic ring systems gives 2-Nal a more elongated shape than 1-Nal. The length difference between 2-Nal and 1-Nal indicates that 2-Nal can penetrate deeper into the membrane compared to 1-Nal. This may be an explanation to why the 2-Nal peptides are more active. The aromatic ring system is expected to be in more close contact with the peptide backbone for 1-Nal, and subsequently the anchoring effect of this amino acid is less pronounced. Kachel *et al.* [20] have studied the anchoring effect of Trp and Tyr analogues, and have found that indole and phenol analogues are located shallower in lipid bilayers than ordinary aromatic hydrocarbons are. We propose that residues able to position themselves deeper into the membrane will impart a higher propensity of the peptide to disrupt the bacterial cell membrane. A deep anchoring of aromatic hydrocarbon residues in the membrane can possibly be more effective in pulling the rest of the peptide into closer contact with the membrane, resulting in increased membrane disruption.

The antibacterial center of LFB has been determined to be RRWQWR [5], a hexapeptide where both of the two Trp residues from the 25-residue peptide are present. The structure of this hexapeptide bound to sodium dodecyl sulfate (SDS) micelles has been determined by NMR spectroscopy [21]. When bound to micelles, the peptide forms an amphipathic structure with the three Arg residues on one side and the two Trp residues on the other side. The Trp side chains are located deeper into the micelles than Arg and Gln. The stability of the structure is suggested to be due to the membrane-anchoring properties of both the Arg and the Trp residues. Recently Schibli *et al.* [22] have observed a similar amphipathic structure for the partial tritrpticin sequence, FPWWWPFL, bound to micelles.

These studies are consistent with Trp being an essential residue for the antibacterial activity of the original LFB peptide [7]. From the positioning of the Trp residues in the micelles, it is clear that these structural elements are important for the membrane interactions of such peptides.

The findings from the Bal and different Nal peptides suggest that neither the hydrogen binding ability nor the amphipathic character of Trp is essential for the remarkable effect of this residue on the antibacterial activity of LFB peptides. The preference of Trp for membrane interfaces is suggested by Yau *et al.* [18] to arise from the aromaticity of Trp (i.e., its π-electron structure and associated electrical quadropole moment). This study rules out simple amphipathic or dipolar interactions as the physical basis for interfacial preference of Trp. It was found that indole analogues upon interaction with phosphatidylcholin membranes reside in the vicinity of the glycerol group, and not in the hydrocarbon core. It is suggested that the interfacial location of Trp may be due to the balance of the hydrophobic effect that tends to drive it out of water, complex electrostatic interactions that favour residing in the hydrated headgroup region, and cohesive repulsion that keeps it out of the hydrocarbon core. Considering these physiochemical aspects, the Nal residues are, because of their aromaticity, expected to have anchoring properties like Trp, but their location in the membrane is expected to be of a deeper kind than that of Trp since they are nonpolar and non-amphipathic [20].

The effect of incorporating benzothienyl and naphthyl instead of indole did not provide insight into whether the size of the aromatic residues is of importance for the antibacterial activity of LFB, as these amino acids are all of comparable size to Trp. To probe the effect of aromatic amino acid side chain size on the activity of LFB derivatives, Phe was introduced as a replacement for Trp. On the interfacial hydrophobicity scale determined by Wimley and White [17], the naturally occurring aromatic amino acids are ranked in the order Trp, Phe and Tyr. This shows that Phe has considerable anchoring properties, despite its small size and lack of amphipathicity.

When incorporating the smaller aromatic amino acid Phe, the peptides showed a remarkable difference in antibacterial activity against the two bacterial strains. The activity against *S. aureus* was lost when replacing Trp by Phe, and the activity against *E. coli* was retained, if not improved, compared to the original LFB peptide. LFB Phe6 and LFB Phe8 showed MIC values of 50 and 25 μg/mL, respectively, against *E. coli*. The loss of activity against *S. aureus* on changing from Trp to Phe clearly shows that for LFB peptides to be active against these bacteria, the aromatic amino acids in the peptide have to be at least the size of Trp. When both Trp residues were replaced with Phe, there was a threefold decrease in activity against *E. coli* also. The poor antibacterial activity of the LFB derivatives containing only Phe instead of Trp shows that for LFB peptides the size of the aromatic residues is important. These findings are consistent with those previously described for Ala analogues of LFB [7]. The replacement of one Trp by Ala results in a total loss of activity against both *S. aureus* and *E. coli*.

We have found in this study that the importance of Trp for the antibacterial activity of LFB peptides is not dependent on the hydrogen binding ability nor on the amphipathicity of the indole side chain. For the antibacterial activity of LFB peptides the size, shape and aromatic character of Trp were found to be the most important features. LFB peptides are believed to be antibacterial by a disruption of the bacterial cell membrane. The cationic residues of the peptide attract it to the bacterial surface by electrostatic interactions. When the peptide comes in contact with the bacteria, the Trp residues are embedded in the membrane surface. Whether the bacteria are killed by the formation of an ion channel, or by the disruption caused by a peptide carpet on the bacterial surface, remains to be elucidated. Regardless of the exact mechanism, the bacterial cell membrane is disrupted upon interaction with the peptides. This disruption will be dependent on the anchoring function, and on the position of the aromatic residues in the membrane. The ability of aromatic hydrocarbons to occupy a deep location in lipid bilayers suggests that LFB derivatives containing such residues will have a higher antibacterial activity.

Acknowledgements

We thank Alpharma A/S (Norway) and the Research Council of Norway (NFR), grant number HS2307/611501, for financial support. We thank Lars H. Vorland and Manuela Krämer at the University Hospital of Tromsø for providing us with the biological data. We thank Morten B. Strøm, Trine Stiberg and Silje Karoliussen for performing some of the peptide syntheses.

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