The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles

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Abstract Lactoferricin B (LfcinB) is a 25-residue antimicrobial peptide released from bovine lactoferrin upon pepsin digestion. The antimicrobial center of LfcinB consists of six residues (RRWQWR-NH_2), and it possesses similar bactericidal activity to LfcinB. The structure of the six-residue peptide bound to sodium dodecyl sulfate (SDS) micelles has been determined by NMR spectroscopy and molecular dynamics refinement. The peptide adopts a well defined amphipathic structure when bound to SDS micelles with the Trp sidechains separated from the Arg residues. Additional evidence demonstrates that the peptide is oriented in the micelle such that the Trp residues are more deeply buried in the micelle than the Arg and Gln residues.

Key words: Lactoferricin B; Antimicrobial peptide; Nuclear magnetic resonance structure; Micelle; Tryptophan

1. Introduction

Lactoferricin B (LfcinB) is a 25-residue peptide fragment released from the N-terminal domain of bovine lactoferrin (residues 17–41) upon pepsin digestion [1,2]. It has been shown to be responsible for the majority of the antimicrobial activity of lactoferrin. Lactoferricin B has antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as yeast [3]. In addition it has been shown to inhibit tumor metastasis in mice [4]. Peptide fragments of lactoferrin, including lactoferricin B, have been isolated from mice fed lactoferrin-enriched milk [5]. Such evidence supports the hypothesis that lactoferrin in milk is naturally digested in the gastrointestinal tract to form antimicrobial peptides, protecting infants as their immune system develops [6].

Studies on an 11-residue fragment of LfcinB (RRWQWRMKKLG) showed that it possesses antimicrobial activity similar to the intact peptide, with reduced hemolytic activity compared to LfcinB. In addition, the authors demonstrated that both the hydrophobic and basic residues were necessary for its antimicrobial activity [7]. Residues 4–9 (RRWQWR) of the lactoferricin peptide ($LfcinB_{4-9}$) have been termed the antimicrobial center of lactoferricin B and when the C-terminal carboxyl group is amidated the hexapeptide has been shown to display antimicrobial activity which compares favorably to LfcinB [8]. Studies with *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* indicate that the 11-residue peptide and the amidated six-residue peptide have the same minimum inhibitory concentration (MIC) as intact lactoferricin B [7,8]. Moreover, recent spectroscopic studies show that LfcinB and its hexapeptide cause similar membrane disturbances in model membranes suggesting that they have a similar mechanism of action (personal communication, Dr. R. Epand, Hamilton, Ont.).

While we have reported the NMR solution structure of the 25-residue lactoferricin B peptide [9], a structure of the peptide in a membrane-like environment has yet to be determined. The elucidation of the structures of membrane proteins by solution NMR spectroscopy methods is possible using SDS micelles [10,11]. We have found that the intact lactoferricin B peptide gives very broad resonances when bound to SDS as well as DPC micelles (unpublished observations), hindering the elucidation of its micelle-bound structure. In this report we describe the structure of the antimicrobial center of the lactoferricin B peptide when bound to SDS micelles, as determined by NMR spectroscopy.

2. Materials and methods

2.1. Materials

The amidated lactoferricin B peptide fragment (residues 4–9 of LfcinB) was synthesized at the Peptide Synthesis Facility at Queen's University (Kingston, Ont.) and was purified by RP-HPLC at the Peptide Synthesis Facility at the University of Calgary. The sodium dodecyl-d_{25} sulfate (SDS-d_{25}) and D_2O were obtained from Cambridge Isotopes Laboratories (Andover, MA), all other reagents were obtained from Sigma (St. Louis, MO).

2.2. Circular dichroism (CD) spectroscopy

The peptide concentration for CD was 10 μM, in a 10 mM Tris buffer, pH 7.2. In micellar samples 35 mM SDS was used, well above the critical micelle concentration. The CD spectra were acquired in a cylindrical cuvette with a 1 mm path length with a Jasco J-175 CD spectrophotometer. The spectra were measured from 255 nm to 185 nm, with a 0.2-nm step resolution at 50 nm/min, a 2-s response time, and a 1-nm bandwidth. Eight scans were collected and averaged with the buffer baseline spectra subtracted.

2.3. NMR spectroscopy

The final peptide concentration was 4 or 10 mM in 90% H_2O/10% D_2O, containing 250 mM perdeuterated SDS, pH 4.8. DSS was added as an internal chemical shift reference compound. Two-dimensional proton NMR spectra were acquired at 500 MHz on a Bruker AMX500 spectrometer at 298 K. The number of data points in the F2 and F1 dimension were 2048 and 512 respectively, with a spectral width of 6024 Hz. The spectra were zero filled to 2K×1K. The data were processed on a Silicon Graphics Indy using NMRPIPE [12].

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Abbreviations: LfcinB, lactoferricin B; $LfcinB_{4-9}$, residues 4–9 of lactoferricin B; SDS, sodium dodecyl sulfate; DPC, dodecylphosphocholine; MIC, minimum inhibitory concentration; CD, circular dichroism; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate; RP-HPLC, reverse phase high performance liquid chromatography; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy

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TOCSY [13] spectra were acquired with a mixing time of 60 ms. NOESY [14] spectra was acquired with mixing times of 50, 100 and 200 ms to check for spin diffusion. The 100-ms mixing time NOESY was used for the assignment and compilation of NOEs. Water suppression was achieved using low power presaturation during the relaxation delay with additional gradient suppression in the NOESY spectra. Hydrogen bonding was monitored via an amide exchange experiment by dissolving a lyophilized NMR sample in 99.9% D_2O and immediately obtaining one-dimensional ^1H NMR spectra at different time intervals.

Distance restraints for structural calculations were determined from the peak volumes in the NOESY spectrum using NMRVIEW 3.1. The restraints were classified as strong, medium or weak, using an internal reference in the peptide. These restraints corresponded to distances of 2.7, 3.5 and 5.0 Å respectively. For atoms where the methyl or methylene protons could not be stereospecifically assigned a pseudoatom correction was used [15].

Samples for NMR experiments in the presence of spin-labeled stearic acid contained the same concentrations as described above. 5-Doxyl stearic acid was added from a 0.1 M stock solution in deuterated methanol to provide a final ratio of SDS to spin-labeled stearic acid of 60:1 [16]. A TOCSY spectrum was obtained using the same conditions as mentioned above.

X-Plor v3.851 [17] was used for all structure calculations according to the protocol previously described [9]. Distance restraints were used to obtain 97 structures calculated using restrained simulated annealing molecular dynamics from an extended starting structure. Of the 97 structures generated, the 14 with the lowest energy were retained.

3. Results and discussion

The CD spectrum of $LfcinB_{4-9}$ (Fig. 1) changes significantly upon addition of SDS with the maximum at 195 nm and the minimum at 205 nm becoming more prominent. In addition, a minor negative peak at 225 nm appears upon the addition of SDS. These three extrema most likely represent the indole transitions of the two tryptophan residues [18], as this spectrum is not indicative of a known protein secondary structure. This increase in the absolute values of mean residue ellipticity may be due to fewer conformers in solution and the stabilization of the structure in the micellar environment.

The proton resonance assignments of $LfcinB_{4-9}$ in SDS micelles were determined using the method of Wüthrich [15]. There was no difference in chemical shifts or NOE crosspeaks

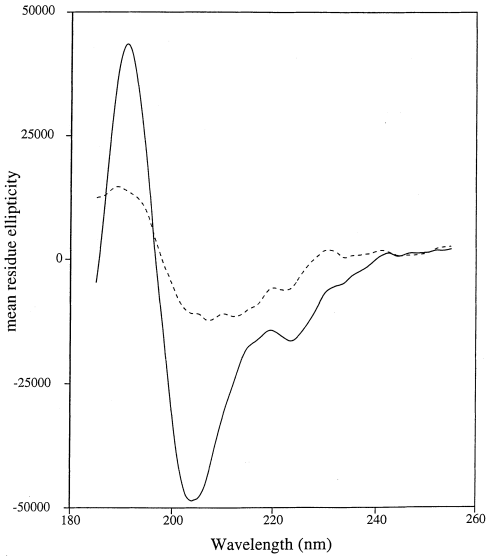

Fig. 1. CD spectra (deg.cm^2/dmol) of 10 μM $LfcinB_{4-9}$ in 10 mM Tris pH 7.4, in the presence (solid line) and absence (dotted line) of 1% SDS.

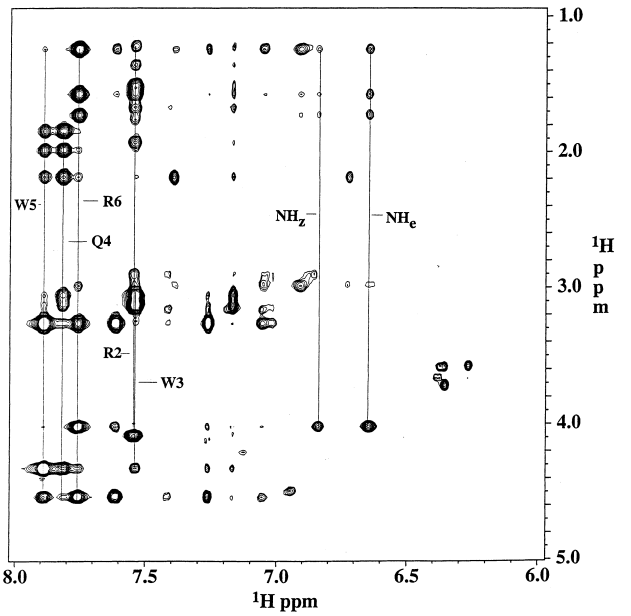

Fig. 2. Fingerprint region of the NOESY spectrum for 10 mM $LfcinB_{4-9}$ in 90% H_2O/10% D_2O, 250 mM SDS, pH 4.8, at 298 K and a mixing time of 100 ms. The amide resonances of residues 2–6 are labeled, as well as the amide protons of the amidated C-terminal carboxyl group.

between the 4- and 10-mM peptide NMR samples hence the 10-mM sample was used for assignment of residues. The proton chemical shifts are presented in Table 1. Many of the chemical shifts observed deviated significantly from expected random coil values. It is known that aromatic residues will cause upfield ring current shifts when spins are above or below the ring plane and downfield when the spins are in the ring plane [15]. The chemical shifts of virtually all of the residues were shifted upfield compared to the random coil chemical shifts. The only residue that was not significantly affected was Arg-1. The most dramatic shifts were seen for the amide protons of residue 2, 3, 4 and 6, all of which were directly above or below one of the two Trp residues as determined by the structure described below. The α protons of residues 3, 4 and 5 were the least affected, as these were generally on the side of the molecule opposite from the indole rings.

A total of 86 NOE crosspeaks were compiled from the 2D NOESY spectrum (Fig. 2) as distance restraints in the structure calculations. Of the 86 distance restraints, 10 were medium range, 44 sequential and 32 intra-residue. Intra-residue restraints that would not affect the structure calculations were excluded. No hydrogen bonds were found based on the 1D ^1H NMR spectroscopy amide exchange experiment. No distance violations larger than 0.3 Å were observed in the final structures, suggesting that a unique structure is formed upon binding to the micelle. The 14 final structures with the lowest energy had an RMSD of 0.947 Å for heavy atoms and 0.265 Å for backbone atoms when all residues were fitted (Fig. 3). There were very few distance restraints obtained for the N-terminal arginine due to chemical shift overlap with other residues. When only residues 2–5 were superimposed the RMSD values were 0.544 Å and 0.082 Å for the heavy and backbone atoms respectively.

The structures obtained did not appear to contain any reg-

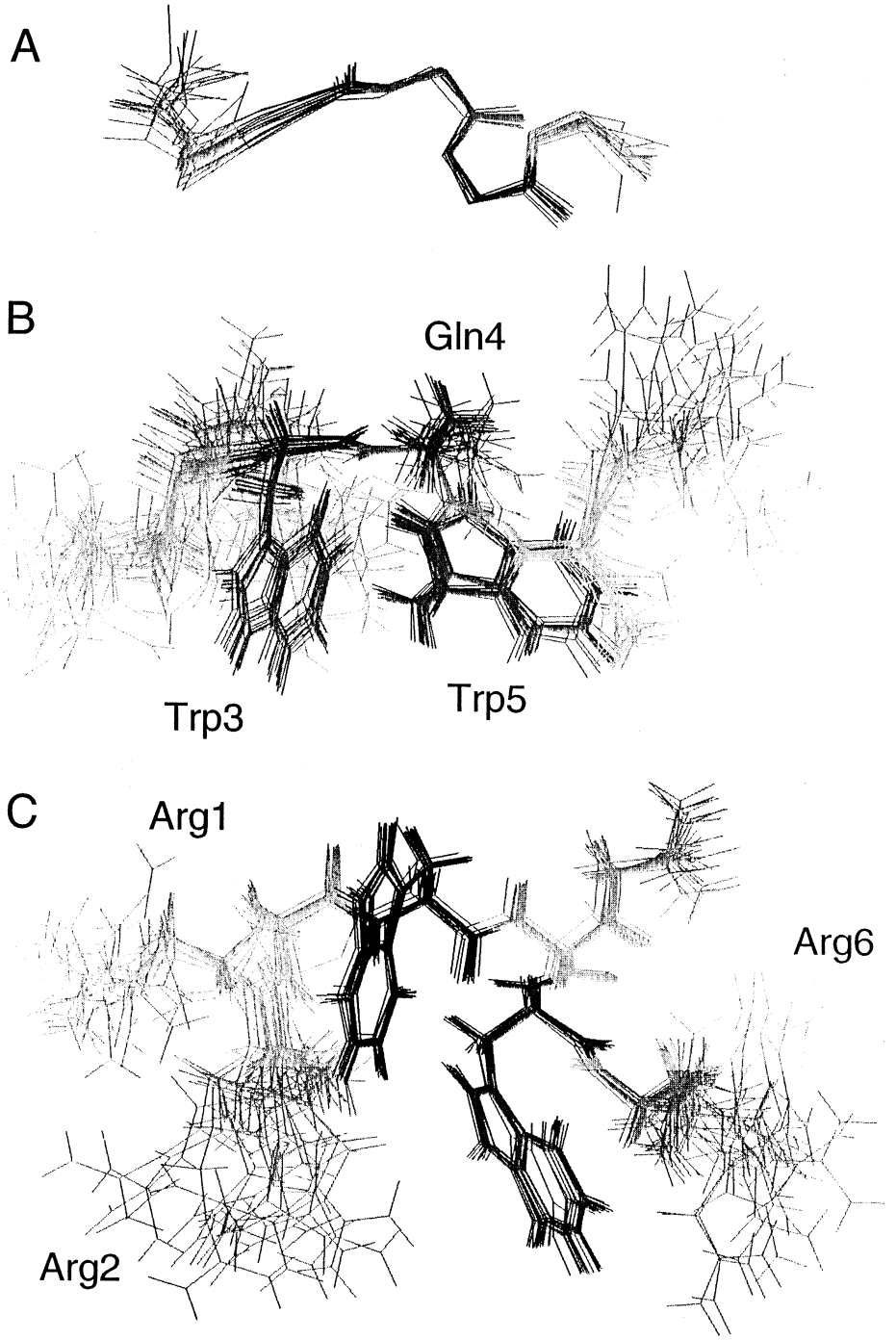

Fig. 3. Final 14 lowest energy structures of $LfcinB_{4-9}$ obtained in SDS-d_{25} micelles with residues 2–5 fitted to the mean structure. A: Backbone of $LfcinB_{4-9}$. B: Side view with Gln pointing out of the page. C: Bottom view of all of the side chains of $LfcinB_{4-9}$. The Trp residues are in black, the Gln residue in dark gray and Arg residues in light gray. Residues are numbered 1–6. The RMSD for backbone atoms is 0.082 Å and 0.544 Å for heavy atoms. The figures were generated using INSIGHTII (Biosym, San Diego, CA).

ular backbone secondary structure. This agrees with both the CD data and the amide exchange experiment. It would be unexpected for a peptide of only six residues to contain a regular secondary structure. The NOE connectivity pattern appears to suggest a half turn for residues 3–6 [15], this is probably coincidental and does not describe a stabilized secondary structure. Although $LfcinB_{4-9}$ does not contain a conventional secondary structure it is not a random coil. The micelle-bound peptide forms a well-defined structure, where the peptide adopts a conformation to obtain the best separation of the hydrophobic and basic residues. In aqueous solution the $LfcinB_{4-9}$ peptide is unstructured as judged from the chemical shifts and the absence of NOE crosspeaks between any of the sidechains in NMR spectra (data not shown).

While there was no well-defined structure for the peptide free in solution, it acquires a unique structure when bound to the SDS micelle. The peptide formed an amphipathic structure with the three Arg residues on one side of the structure

and the two Trp residues on the other with the Gln centrally located (Figs. 3 and 4). The formation of an amphipathic structure has been postulated to be a requirement for cationic antimicrobial peptides (recent reviews by [19,20]), and has been observed for helical peptides like cecropin A [21] and magainin 2 [22] and β-sheet peptides such as insect defensin A [23].

The structure of the 25-residue LfcinB peptide in solution was determined to have an amphipathic structure with the Trp residues separated from the positively charged Arg and Lys residues [9]. While the structure of the micelle-bound hexapeptide, $LfcinB_{4-9}$, is also amphipathic, the two structures can not be superimposed. This is due to the fact that the sidechains of the C-terminal β-sheet of LfcinB interact with the sidechains of the N-terminal β-sheet, specifically Ile-18 intercalates between Trp-6 and Trp-8 [9], disrupting the indole interactions which are clearly evident in $LfcinB_{4-9}$.

A TOCSY spectrum of $LfcinB_{4-9}$ in SDS micelles in the presence of 5-doxyl stearic acid was used to study the orientation of the peptide in the micelle [16]. All of the resonances in the TOCSY spectrum were affected by the presence of the spin label in the micelle (data not shown), however the Trp peaks were broadened the most, followed by Arg, and the Gln resonances were the least affected. This agrees with the structure that was determined for $LfcinB_{4-9}$ where the two Trp residues are more deeply buried in the micelle with the Arg and Gln sidechains more solvent-exposed.

The structural stability of $LfcinB_{4-9}$ in the SDS micelles appears to be due to the anchoring nature of many of the residues in the peptide. The three Arg residue sidechains and the N-terminus probably interact electrostatically with the negatively charged head groups of the SDS molecules. Tryptophan is known to position peptides at the membrane interface [24] such that they act as membrane anchors [25]. The two Trp residues of $LfcinB_{4-9}$ reside within the micelle and may act as hydrophobic anchors. This provides the six-residue antimicrobial center of lactoferricin with five residues which will act to position the peptide at the bilayer surface, therefore it is perhaps not surprising to find that such a small peptide will form a stable folded structure in micelles.

Yau et al. recently demonstrated that Trp analogues will bind in the interface of POPC bilayers, residing amongst the acyl chain carbons closest to glycerol [26]. This confirms previous data which suggested that Trp residues [27] and indole

Table 1
Proton chemical shifts (ppm) relative to DSS for LfB_{4-9} in the presence of 250 mM SDS-d_{25} at 298 K and pH 4.8

	NH	αH	βH	γH	Others
Arg-1		4.01	1.74, 1.94	1.66	δH: 3.17 εH: 6.88
Arg-2	7.55	4.08	1.53, 1.57	1.22, 1.36	δH: 2.93 εH: 7.18
Trp-3	7.54	4.70	3.05, 3.13	1H: 9.73 4H: 7.54 6H: 7.06	2H: 7.16 5H: 7.00 7H: 7.41
Gln-4	7.82	4.33	1.85, 2.00	2.19	NH: 6.72, 7.40
Trp-5	7.89	4.54	3.26	1H: 9.83 4H: 7.62 6H: 7.05	2H: 7.24 5H: 7.02 7H: 7.39
Arg-6	7.76	4.02	1.58, 1.73	1.24	δH: 2.99 εH: 6.91
$CONH_2$	NH_e: 6.64 NH_z: 6.84				

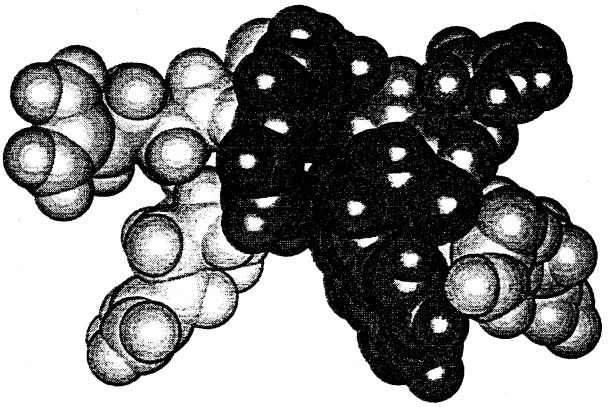

Fig. 4. Bottom view of a van der Waals surface plot of $LfcinB_{4-9}$ in SDS-d_{25} micelles. The Trp residues are in black, the Gln residue in dark gray and the three Arg residues in light gray. This figure was generated using INSIGHTII (Biosym, San Diego, CA).

[28] both have a preference for the hydrocarbon-water interface of membranes. The importance of tryptophan as a membrane interface binding moiety has been observed for various peptides such as gramicidin A [29], galanin [16] and mellitin [30]. There is a tryptophan-rich class of antimicrobial peptides including indolicidin [31] and tritrpticin [32], suggesting that tryptophan may play a pivotal role in membrane binding. In addition Trp- and Arg-rich hexamer peptides were identified using combinatorial chemistry which had similar antimicrobial activities to magainin 2 and cecropin [33]. While the intact 25-residue lactoferricin B peptide would not be included in such a class, the $LfcinB_{4-9}$ peptide studied here should be considered a tryptophan-rich peptide, as this amino acid constitutes one-third of the residues.

While the structure of the peptide and its orientation in a micelle have been determined from this work, the mechanism of its bactericidal activity remains to be investigated. Various lytic mechanisms have been proposed for amphipathic α-helical antimicrobial peptides (for recent reviews see [34,35]). Since $LfcinB_{4-9}$ has an amphipathic structure it is possible that it has a related mechanism. The proposed lytic mechanisms for antimicrobial peptides include the channel forming models proposed for magainin, cecropin and almethicin [35]. The formation of bundles of amphipathic molecules that form a pore with a hydrophilic surface is doubtful for $LfcinB_{4-9}$ due to its short length. It has also been suggested that small peptides may still form bundles by forming head-to-tail structures [36], this is also unlikely since there are no electrostatic or hydrophobic interactions to stabilize such a structure. Another possible mechanism is the induction of monolayer curvature strain by amphipathic peptides [34,37]. It is probable that $LfcinB_{4-9}$ is inducing membrane curvature strain such as the model lytic peptide 18L [38] and as has been recently suggested for magainin 2 [39].

In conclusion, $LfcinB_{4-9}$ has been found to form a stable amphipathic structure in SDS micelles, with the Trp sidechains located deeper in the micelle than the Arg and Gln residues. The stable structure appears to be due to the membrane anchoring properties of the Trp and Arg residues. Further work must be performed to elucidate the structure of the 25-residue peptide in a membrane mimicking environment and to determine the lytic mechanism of these two forms of lactoferricin B.

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