Structures of Human Host Defense Cathelicidin LL-37 and Its Smallest Antimicrobial Peptide KR-12 in Lipid Micelles

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As a key component of the innate immunity system, human cathelicidin LL-37 plays an essential role in protecting humans against infectious diseases. To elucidate the structural basis for its targeting bacterial membrane, we have determined the high quality structure of $^{13}$C,$^{15}$N-labeled LL-37 by three-dimensional triple-resonance NMR spectroscopy, because two-dimensional $^1$H NMR did not provide sufficient spectral resolution. The structure of LL-37 in SDS micelles is composed of a curved amphipathic helix-bend-helix motif spanning residues 2–31 followed by a disordered C-terminal tail. The helical bend is located between residues Gly-14 and Glu-16. Similar chemical shifts and $^{15}$N nuclear Overhauser effect (NOE) patterns of the peptide in complex with dioctanoylphosphatidylglycerol (D8PG) micelles indicate a similar structure. The aromatic rings of Phe-5, Phe-6, Phe-17, and Phe-27 of LL-37, as well as arginines, showed intermolecular NOE cross-peaks with D8PG, providing direct evidence for the association of the entire amphipathic helix with anionic lipid micelles. The structure of LL-37 serves as a model for understanding the structure and function relationship of homologous primate cathelicidins. Using synthetic peptides, we also identified the smallest antibacterial peptide KR-12 corresponding to residues 18–29 of LL-37. Importantly, KR-12 displayed a selective toxic effect on bacteria but not human cells. NMR structural analysis revealed a short three-turn amphipathic helix rich in positively charged side chains, allowing for effective competition for anionic phosphatidylglycerols in bacterial membranes. KR-12 may be a useful peptide template for developing novel antimicrobial agents of therapeutic use.

The growing drug resistance problem of pathogenic bacteria with traditional antibiotics calls for an urgent search for a new generation of antimicrobial agents. Antimicrobial peptides are ancient and potent weapons of the innate immunity of all life forms (1–4). The recently updated Antimicrobial Peptide Data base collects more than 1228 such peptides (5). In mammals, defensins and cathelicidins are the two major families of antimicrobial peptides. While several cathelicidins were found in animals such as sheep, cow, and pig, only one cathelicidin was identified in humans (6). The precursor proteins of the cathelicidin family share a highly conserved N-terminal "cathelin" domain, but have a highly variable C-terminal antimicrobial region. Upon bacterial insult, human cathelicidin LL-37 (named based on the first two amino acids in the sequence followed by the number of residues in the peptide) is released by proteases from its precursor hCAP-18 (i.e. human cationic antimicrobial protein, ~18 kDa). The importance of this host defense peptide to human health is now firmly established. Patients lacking this molecule are more susceptible to infections (7). While cathelicidin knock-out mice are more readily infected (8), expression of additional cathelicidins protects the animals from infection (9). LL-37 also associates with lipopolysaccharides (or endotoxin) and protects rats from sepsis caused by bacteria (10). LL-37 is also reduced in cystic fibrosis airways as a result of direct interaction with DNA and filamentous F-actin (11). As a consequence, there is a high interest in developing novel peptides of therapeutic value based on LL-37.

To provide insight into the mechanism of action of LL-37, biochemical and biophysical studies lent support to the membrane targeting of the peptide. Oren et al. (12) found that LL-37 self-associated when bound to zwitterionic lipids, but dissociated into monomers in the presence of negatively charged vesicles. CD studies indicated a helical conformation of LL-37 upon binding to membrane-mimetic models (13). Solid-state NMR studies indicated that LL-37 is located on the surface of lipid bilayers (14). Using the monolayer of phospholipids, LL-37 was demonstrated to have preferential interactions with anionic phosphatidylglycerols (PGs)$^2$ (15). This lipid selection is essential for selective targeting of anionic bacterial membranes by cationic antimicrobial peptides.

The three-dimensional structure of intact LL-37 in complex with bacterial membranes would be invaluable to understanding the mechanism of action, but was not available prior to our

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$^2$The abbreviations used are: PG, phosphatidylglycerol; D8PG, dioctanoylphosphatidylglycerol; DPC, dodecylphosphocholine; HSQC, heteronuclear single-quantum coherence spectroscopy; MIC, minimum inhibitory concentration; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; r.m.s.d., root mean square deviation.
study. Although homonuclear two-dimensional NMR techniques set the record of structural determination of micelle-bound peptides and proteins up to ~50 residues (16), we met difficulty in structural studies of this 37-residue membrane peptide in complex with SDS micelles due to heavy spectral overlap (Fig. IA). Thus, it is necessary to utilize three-dimensional NMR techniques. For this purpose, isotope-labeled polypeptides are required. Direct expression of LL-37 in yeast gave a very low level of the peptide (17). Using bacterial expression systems, we obtained 1.7 mg of recombinant LL-37 from one liter of culture (18), whereas Moon et al. (19) reported 0.3 mg. Subsequent studies revealed that the LL-37-containing fusion protein was essentially uncleaved by thrombin at a site adjacent to LL-37 (probably due to peptide aggregation), but could be readily digested by formic acid. Our continued improvements not only simplified the purification protocol for LL-37 but also improved the peptide yield (reviewed in Ref. 16), opening the door to structure and dynamics studies of LL-37 by three-dimensional NMR spectroscopy. As a consequence of formic acid cleavage at the Asp-Pro site, recombinant LL-37 obtained in this manner retained an extra proline residue at the N terminus. We demonstrated that the recombinant LL-37 we obtained had an identical antibacterial activity to the synthetic peptide corresponding to the native sequence (18). Therefore, this form of recombinant LL-37 is useful for structural analysis.

Because of the complex nature of bacterial membranes, structural studies of membrane-associated peptides and proteins by solution NMR are usually performed in membranes-mimetic micelles (16). This is because the faster the tumbling of the peptide/lipid complex, the narrower the spectral linewidth. Here we report the three-dimensional structure of recombinant LL-37 in SDS micelles. To provide additional insight into the interactions of human cathelicidin with anionic PGs, we also conducted the study in the presence of D8PG, a new bacterial membrane model for solution NMR (16). Using synthetic peptides, we also report the identification and structural studies of the shortest antimicrobial peptide, KR-12, corresponding to residues 18–29 of LL-37. Selective toxicity of KR-12 makes it a promising candidate for developing novel and potent antimicrobial agents.

**EXPERIMENTAL PROCEDURES**

**NMR Spectroscopy**—15N- or 15N,13C-labeled recombinant LL-37 was expressed and purified using the established protocol (20). The LL-37/SDS complex contains ~0.5 mM peptide and 80-fold of deuterated SDS (Cambridge Isotope Laboratories). A complex was also made between 15N- or 15N,13C-labeled LL-37 (~0.5 mM) and protonated D8PG (>98%, Avanti lipids, AL) at a molar ratio of 1:30. To facilitate peptide-lipid NOE observations, 2 mM synthetic LL-37 (>95% purity, Genemed Synthesis, TX) was co-solubilized with 10 mM D8PG. A similar sample was made for KR-12 in D8PG. The pH of all NMR samples, containing 10% D2O, was measured directly in the NMR tubes using a micro-pH electrode (Wilmad-Labglass).

NMR data were recorded on a Varian INOVA 600-MHz NMR spectrometer equipped with a triple-resonance cryogenic probe with a z-axis gradient capability. In two-dimensional 1H NMR spectra, the spectral width in both dimensions was 8510.6 Hz. In the case of two-dimensional HSQC spectra, the sweep width for the 15N dimension was typically set to 2200 Hz with 100 increments. For natural abundance HSQC, 30 increments were collected in the 15N dimension with 256 scans each. For temperature coefficient measurements, HSQC spectra of LL-37 were recorded at every 5 degrees from 15 to 30 °C. The temperature coefficient (in ppm × 10⁻³/°C) for each backbone amide proton was calculated by linear regression of chemical shifts versus temperature. Residue-specific 15N(1H) NOE values for 15N-labeled LL-37 in SDS or D8PG micelles were measured using two-dimensional (1H, 15N) correlated spectroscopy with and without proton saturation as described (21).

For signal assignments, several double- and triple-resonance NMR experiments, including HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA, HNCO, C(CO)NH, H(CCO)NH, HNHA, HBHA(CO)NH, HCCCH-COSY, and HCCCH-TOCSY (22), were performed using 13C,15N-labeled LL-37 as detailed previously (23). Typically, these three-dimensional experiments were recorded with sweep widths/increments of 1,000 Hz/28 for 15N, 12067.8 Hz/60 for aliphatic 13C, and 3770 Hz/40 for carbonyl carbon in the indirect dimensions, and with a spectral width of 8510.6 Hz and 1024 complex points in the 1H-detected dimension, respectively. The carriers for 1H, 15N, and 13C were positioned at 4.67, 118.2, and 47.3 ppm, respectively. Chemical shifts were referenced as described (23). Data were processed on a Silicon Graphics Octane work station using NMRPipe (24) and analyzed by PIPP (25).

**Structure Calculations**—For structural calculations of SDS-bound LL-37, the major restraints were derived from three-dimensional NOE spectra (26). For D8PG-bound KR-12, distance restraints were obtained from two-dimensional NOESY spectra. The cross peaks were integrated by PIPP (25) and converted to distance restraints 1.8–2.8, 1.8–3.8, 1.8–5.0, and 1.8–6.0 Å corresponding to strong, medium, weak, and very weak types of NOE peaks, respectively. Based on 1Hα, 15N, 13Ca, 13Cβ, and 13C carbonyl chemical shifts, backbone angles of micelle-bound LL-37 were predicted by using an updated version of TALOS (27). Hydrogen bond restraints for the structured region were derived from temperature coefficients as described (21). For KR-12 in D8PG micelles, backbone angles were predicted based on 1Hα and natural abundance 15N chemical shifts (16). An extended covalent structure was used as starting coordinates. An ensemble of structures was calculated by using the simulated annealing protocol in the Xplor-NIH program (28). Structures were accepted based on the following criteria: no NOE-derived distance violations greater than 0.20 Å, back dihedral angle violations less than 2°, r.m.s.d. for bond deviations from ideality less than 0.01 Å, and r.m.s.d. for angle deviations from ideality less than 5°. The structures were viewed and analyzed using PROCHECK (29) and MOLMOL (30).

**RESULTS**

**Solution Structure of Human LL-37 in Complex with SDS Micelles**—The 1H signals of LL-37 in SDS are poorly dispersed in the two-dimensional NOESY spectrum (Fig. 1A), but are well separated in different two-dimensional planes of the three-dimensional NOESY spectrum (Fig. 1B). Sequential 1H, 13C, and
The necessity of three-dimensional NMR spectra for structural determination of human LL-37 in SDS micelles. The poor spectral resolution as illustrated by a portion of the two-dimensional NOESY spectrum (mixing time 100 ms) of synthetic LL-37 made it impractical to determine the structure in SDS by two-dimensional NMR (A). In contrast, spectral resolution became superb after separating the crowded two-dimensional data onto multiple two-dimensional planes of the three-dimensional 15N-separated NOESY spectrum of LL-37 collected at a peptide/SDS ratio of 1:80, pH 5.4 and 30 °C. In B, a two-dimensional plane at the 15N chemical shift of 120.34 ppm of the three-dimensional NOESY is given to illustrate the excellent resolution.

**TABLE 1**

| Structural statistics of human LL-37 and KR-12 bound to micelles |
|---------------------------------------------------------------|
| **Structural restraints** | **LL-37 in SDS** | **KR-12 in D8PG** |
|--------------------------|------------------|------------------|
| NOE restraints (total)    | 345              | 86               |
| Intra-residue            | 124              | 17               |
| Sequential               | 128              | 37               |
| Short range              | 94               | 32               |
| Backbone angles (d/\(\phi\)) | 58         | 20               |
| Hydrogen bonds           | 19               |                  |
| **Structural quality**   |                  |                  |
| Backbone r.m.s.d. (Å)\(^a\) | 0.69      | 0.24             |
| Distance violations (Å)   | <0.2             | <0.2             |
| Dihedral angle violations | <2\(^\circ\)    | <2\(^\circ\)    |
| **Ramachandran plot**\(^b\) |                  |                  |
| Residues in the most favored region | 93.8%       | 90.0%            |
| Residues in the additional allowed region | 6.2%        | 10.0%            |

\(^a\) Predicted by the updated version of the TALOS program (27).

\(^b\) Calculated by MOLMOL (30). For LL-37, residues 2–30 of an ensemble of 28 structures were superimposed; for KR-12, residues 20–28 of an ensemble of 20 structures were superimposed.

\(^c\) Calculated by PROCHECK (29).

15N signal assignments were then achieved by using triple-resonance NMR experiments (22) using isotope-labeled samples (23). TALOS (27) analysis of 1H, 15N, 13Cα, 13Cβ, and 13C carbonyl chemical shifts of micelle-bound LL-37 identified a helical region covering residues 2–30. No consensus angles were predicted for residues 31–36. The three-dimensional structure of LL-37 in complex with SDS was determined based on the following NMR restraints: 345 NOE-derived distance restraints, 58 chemical shift-derived backbone angle restraints, and 19 temperature coefficient-derived hydrogen-bond restraints (Table 1). Fig. 2A presents an ensemble of 28 structures of LL-37 accepted based on the criteria defined under “Experimental Procedures.” The r.m.s.d. is 0.69 Å when the backbone atoms of residues 2–30 are superimposed. According to PROCHECK analysis (29) of the ensemble of structures deposited into the Protein Data Bank, 93.8% of the residues are located in the most favored region of the Ramachandran plot and 6.2% are located in the additionally allowed region, indicating high quality. A ribbon diagram of the structure (Fig. 2B) is generated using MOLMOL (30). The three-dimensional structure of LL-37 consists of a well-defined long helix covering residues 2–30 with the remaining C-terminal residues disordered. The overall structural pattern of LL-37 in SDS micelles agrees well with both heteronuclear NOE measurements (Fig. 3A) and the secondary chemical shift plot (Fig. 3B). The former indicates that residues 2–32 are ordered and the C terminus is mobile, while the latter indicates a helical region covering residues 2–31 of LL-37. A further examination found that the amphipathic helix is curved with a bend between residues Gly-14 and Glu-16 (Fig. 2, B and C). Residues 14–16 as the bend are consistent with the 1H secondary chemical shift plot (Fig. 3B), where residues 14 and 16 showed near zero or positive deviations. We propose that the hydrophobic packing between residues Ile-13 and Phe-17 plays an important role in causing the helical bend in the structure of LL-37. Aligned on the concave surface is a train of hydrophobic side chains: Leu-2, Phe-5, Phe-6, Leu-13, Phe-17, Ile-20, Val-21, Ile-24, Phe-27, Leu-28, and Leu-31. Note that a hydrophilic residue Ser-9 is also located on the hydrophobic surface (Fig. 2B), leading to a division of the hydrophobic surface into two regions. The aromatic-aromatic stacking between Phe-5 and Phe-6 (Fig. 2B), also observed in the structure of the N-terminal fragment of LL-37 (31), may be responsible for the upfield shift of the H3 protons of Phe-5 at 6.92 ppm.

Structure of LL-37 in D8PG Micelles Is Similar to That in SDS—To further understand the effect of different micelles on the structure, we also performed NMR studies of LL-37 in D8PG (32–34), which has the same lipid head group as the major anionic PGs in bacterial membranes. In D8PG, spectral quality of 15N-labeled LL-37 was poor at low temperatures. However, a well-resolved spectrum was obtained at 50 °C (see
NMR Structure of Human LL-37

[Image 60x398 to 288x733]

FIGURE 3. Heteronuclear NOE, chemical shift plots of LL-37 and its interaction with D8PG. A, $^{15}$N NOE values of LL-37 measured in SDS (peptide/SDS ratio 1:80, pH 5.4, 30 °C). B, $^1$H secondary shift plot of LL-37 in SDS. Secondary shifts were calculated by taking the differences between the measured chemical shifts of the peptide and those random-coil values (46). C, $^1$H secondary shift plot of $^{15}$N-labeled recombinant LL-37 in D8PG (peptide/D8PG 1:30, pH 5.4, 50 °C). D, intermolecular NOE cross peaks between synthetic LL-37 (2 mM) and D8PG at a peptide/D8PG molar ratio of 1:5, pH 5.4 and 30 °C. The corresponding one-dimensional slice was taken to show weak NOE cross peaks between arginines and D8PG (pointed by arrows).

supporting information, see supplemental Fig. S1). An estimation of the correlation time ($\tau_c$) of the complex based on the ratio of the $^{15}$N $T_1$/$T_2$ relaxation times of the entire amide region (35) revealed that the $\tau_c$ of the LL-37/D8PG complex at 50 °C was 6.9 ns (similar to 7.0 ns for an 18-kDa globular protein IIA $^{\text{Bchain}}$ in solution at 35 °C), while the $\tau_c$ values in SDS are 7.0, 6.83, and 6.36 ns at 25, 35, and 45 °C, respectively. Clearly, the NMR-derived correlation time of LL-37 in D8PG at 50 °C is comparable to that in SDS at ~30 °C, indicating that the LL-37/SDS complex tumbles faster than the LL-37/D8PG complex in aqueous solution at the same temperature. Based on the chemical shift assignments of LL-37 in D8PG, we have also obtained the $^4$H$\alpha$ secondary shifts (i.e., the measured shifts minus the random-coiled shifts). The plots of LL-37 obtained in SDS (Fig. 3B) and in D8PG (Fig. 3C) are remarkably similar, indicating a helical structure from residues 2 to 31 in both systems. Furthermore, TALOS analysis of $^1$H$\alpha$, $^{15}$N, $^{13}$C$\alpha$, $^{13}$C$\beta$, and $^{13}$C carbonyl chemical shifts of LL-37 in D8PG led to the definition of the helical region covering residues 2–30, i.e., also identical to that found in SDS. At 50 °C, the overall trend of heteronuclear NOE values of human cathelicidin in D8PG (not shown) is also similar to that in SDS (Fig. 3A). We conclude that the structures of LL-37 in SDS and D8PG micelles are very similar if not identical.

The introduction of protonated D8PG, based on the comparison of a series of PGs (33, 34), also provides a good opportunity to measure the interactions between cationic LL-37 and anionic PGs. The through-space dipole-dipole interactions between protons (<5 Å) are manifested in intermolecular NOE spectra (16, 36). Such NOE cross peaks were found to build up as normal intramolecular NOE peaks, indicating direct dipole-dipole interactions (26, 33). As shown in Fig. 3D, the aromatic rings of Phe-5, Phe-6, Phe-17, and Phe-27 of LL-37 on the concave hydrophobic surface (Fig. 2C) all displayed NOE cross peaks with the D8PG C3-C7 protons at 1.23 ppm. Similar cross peaks were also observed when one-dimensional slices were taken in the two-dimensional spectrum corresponding to lipid signals at 2.34 ppm (C2-H) and 5.25 ppm (H4$\beta$). These lipid signals possess unique chemical shifts and do not overlap with the peptide signals under investigation. As there are NOE cross peaks from the peptide to both lipid head group and acyl chain protons, LL-37 binds to the interfacial region of the lipid micelles. Furthermore, slice analysis facilitates the viewing of some weaker NOE cross peaks such as those between arginine side-chain protons and D8PG (pointed with arrows in Fig. 3D). Such Arg-PG cross peaks, albeit being weak, were also detected using an engineered LL-37 peptide (33), providing long-desired evidence for Arg-PG interactions. Observations of similar NOE cross peaks from lysines to PGs would be more difficult due to fast exchange of the side chain NH protons with solvents.

Identification and Structural Determination of the Shortest Antibacterial Peptide Derived from LL-37—The existence of a helical bend between residues 14–16 in LL-37 allows us to classify micelle-bound human cathelicidin into three structural and functional regions. The N-terminal region (residues 1–13, labeled as I in Fig. 2B) has been implicated in chemotaxis (37), in peptide oligomerization, in conferring proteolysis resistance, and in hemolytic activity of LL-37 (12, 13). The C-terminal region of LL-37 (residues 32–37, III in Fig. 2B) is disordered and mobile in complex with SDS, D8PG, or lipopolysaccharides (see online supporting information, supplemental Fig. S1B) and thus plays little role in targeting bacterial membranes. Our previous study found that these C-terminal residues participate in tetramer formation of LL-37 at physiological pH (20). It remains to be established whether the C-terminal tail of LL-37 is involved in other biological functions.

Numerous studies verified that the middle region (residues 17–31, II in Fig. 2B) contains the primary antimicrobial region of LL-37 (37–40). Using NMR, we previously mapped a core antimicrobial and anticancer region to residues 17–29 (FK-13) of LL-37 (31). However, FK-13 remained toxic to human cells. Recently, we demonstrated that FK-13 is truly the smallest anti-HIV region of LL-37, since further deletion of residue F17 from tetramer formation of LL-37 at physiological pH (20). It remains to be established whether the C-terminal tail of LL-37 is involved in other biological functions.
LL-37, we have tested the antibacterial activities of additional synthetic peptides (Fig. 4) using the standard microdilution method as described (42). These 10–12-residue peptides, with C-terminal amidation, are named in the same manner as their parent peptide LL-37. For instance, KR-12 starts with amino acids KR and contains 12 residues. We found KR-12 (MIC = 40 \mu M) is as active as LL-37 or FK-13 (31) against Escherichia coli K12. However, KR-11 is only weakly active (MIC ~160 \mu M), and KR-10 and RI-10 are both inactive (MIC >160 \mu M). Therefore, this study has identified KR-12 as the smallest antibacterial peptide of LL-37. Different from FK-13, KR-12 showed no cytotoxicity to human cells till 100 \mu g/ml (or 63 \mu M) (41). This is an important finding as we only achieved selective toxicity previously for an LL-37-based peptide by partially incorporating D-amino acids (31).

To provide insight into the mechanism of action, we also determined the three-dimensional structure of KR-12 in complex with D8PG by utilizing the improved two-dimensional NMR method (33). A summary of the structural restraints and quality data is also provided in Table 1. A short helical region is found between residues 20 and 28 (Fig. 5). This three-turn amphipathic helix is rich (41.6%) in cationic side chains. Such a positive charge cluster (Fig. 5, blue) is also evident in the structure of intact LL-37 (Fig. 2C).

DISCUSSION

The interest in LL-37 has been growing worldwide as evidenced by an exponential increase in related literature in PubMed (38). To improve our understanding of the structure and function of this important host defense cathelicidin, my laboratory has been interested in the structural determination of LL-37 and its complexes with other molecules. Previously, we determined the structures of a few fragments of LL-37 using synthetic materials (31, 33, 34). In this study, we completed the structural determination of intact LL-37 using isotope-labeled samples. While we found insufficient spectral resolution for structural studies of LL-37 in SDS by two-dimensional NMR (Fig. 1A), Porcelli et al. (43) reported the structure of synthetic LL-37 in DPC micelles. Interestingly, the structure in DPC determined based on two-dimensional NMR spectra differs in several aspects from our structure in SDS determined using three-dimensional NMR spectra. First, the well-structured region includes residues 4–33 in DPC, but covers residues 2–31 in SDS. Thus, both the helix starting and ending residues in the two structures of LL-37 are different. Second, a helix-break-helix motif was found in DPC micelles with the break at residue Lys-12. In contrast, we found a continuous helical structure with a helical bend between residues 14–16. Third, both the N and C termini of LL-37 were found to be flexible in DPC micelles. In contrast, our heteronuclear NOE data for LL-37 in either SDS or D8PG micelles indicate that the N-terminal region is as rigid as the middle region, and only the C-terminal region is dynamically mobile (Fig. 3A). These structural differences may result from the different micelles used (DPC versus SDS) and/or the NMR spectral resolution (two-dimensional versus three-dimensional). Future structural studies of intact LL-37 in DPC by three-dimensional NMR may resolve this issue.

Tossi and co-workers (44) have recently determined more than a dozen cathelicidin sequences. Fig. 6 shows the cathelicidin sequences of a few representative primates. Relative to human LL-37, the sequence similarities of primate cathelicidins range from 100% (chimpanzee), 91–96% (the Great Apes and the Old World Monkeys), to 78–84% (the New World Monkeys). The overall amphipathic pattern is retained in these peptides with the major difference being the net charge. Furthermore, CD studies confirmed that these 37-residue homologous cathelicidins formed a helical structure in the presence of SDS (44). Therefore, the three-dimensional structure of human...
LL-37 determined here in SDS (Fig. 2) should be applicable to those highly homologous cathelicidins. This sequence comparison (Fig. 6) also provides additional insight into the structure and function of human LL-37. First, in the amphipathic structure of LL-37, the hydrophobic surface is interrupted by a unique hydrophilic residue Ser-9 (Fig. 2B). In homologous primate sequences (with the exception of chimpanzee) (44), however, Ser-9 is substituted by either an Ala (in 14 sequences) or Val (in 5 sequences) residue (Fig. 6, in bold). Such a mutation, if made in LL-37, would render the hydrophobic surface of the structure continuous. Future mutagenesis studies are required to uncover the unique functional roles of Ser-9 in LL-37. Second, a helical bend is found in the LL-37 structure between residues 14–16 (Fig. 2B). It is of outstanding interest to note that any residue between 14 and 16 can be a glycine in the homologous cathelicidins (Fig. 6, underlined). Furthermore, two of the three residues in that region are glycines in the cathelicidin sequence of capuchin (a New World monkey), while all three residues are glycines in the bend sequences of the rhesus macaque monkey and several other primate cathelicidins (44). As small glycines may introduce flexibility into peptide chains, this observation is in accord with our definition of residues 14–16 as the bend between the two helical regions of LL-37 (Fig. 2B). We do not exclude a possible conformational change with an increase in the number of glycines in that region of primate cathelicidins. Third, through our study, it is established that the four aromatic rings (Phe-5, Phe-6, Phe-17, and Phe-27) of LL-37 are all involved in micelle binding (Fig. 7A) as indicated by peptide-lipid NOE cross peaks (Fig. 3D). Among them, however, Phe-27 might be more important in membrane binding, because it is the only Phe residue that is highly conserved in homologous cathelicidins (Fig. 6, italicized). Furthermore, F27 is the only aromatic residue that remains in the smallest anti-microbial peptide KR-12 derived from LL-37. Indeed, aromatic Phe residues are more common than either Trp or Tyr in the 1228 antimicrobial peptides collected in the Antimicrobial Peptide Database (5). Interestingly, a group of Phe aromatic rings also exists in the concave interface of glucose-specific enzyme IIA<sup>Glc</sup> of <i>E. coli</i> required for the recognition of its up- and downstream partner proteins (45) for bacterial signal transduction and for carbohydrate utilization and regulation (Fig. 7B). Thus, interfacial Phe residues are not limited to protein-lipid interactions and can be important in protein-protein interactions as well.

The mechanisms of action of cationic antimicrobial peptides are in general poorly understood. In the case of LL-37, previous studies proposed a carpet model for the action of LL-37 (12). The surface location of LL-37 in lipid bilayers is supported by solid-state NMR (14) and also consistent with intermolecular NOE observation here in lipid micelles. Peptide-lipid NOE patterns also support the micelle surface location of KR-12. The detection of the Arg-PG interactions using both intact LL-37 (Fig. 3D) and its fragments (33), including KR-12, prompts us to propose a different model for the mechanism of action. The positive charge cluster of KR-12 (Fig. 5, C and D) may serve as a “magnet” that effectively attracts, and competes for, negatively charged PGs in bacterial membranes. Such a re-distribution of anionic lipids in bacterial membranes would cause the formation of “PG-rich domains”, which Epand and co-workers (46) have observed using peptide analogs. The loss of anionic PGs to cationic antimicrobial peptides can have a global impact on bacteria. For example, it would hinder bacterial signal transduction that requires PGs for membrane targeting of amphitropic proteins such as IIA<sup>Glc</sup> of <i>E. coli</i> (32). It would also influence the normal functions of other membrane-bound proteins such as voltage-dependent potassium channels that also require proper Arg-PG interactions (47). These effects of antimicrobial peptides would disturb the membrane physiology of bacteria, leading to cell growth inhibition and death.

In summary, we have determined the three-dimensional structure of human LL-37 by three-dimensional heteronuclear NMR spectroscopy. The structures of LL-37 are similar in SDS and D8PG micelles (this study), but differ from that in DPC determined by two-dimensional NMR techniques (43). Our structure of LL-37 provides novel insight into the potential functional roles of human LL-37 and will stimulate new experiments to test new hypotheses. It also serves as a useful model to understand the structure-activity relationship of homologous primate cathelicidins as well as the fragments of LL-37. Finally, the identification of the smallest and selective antibacterial region of human LL-37 may facilitate the engineering of novel antimicrobial peptide analogs.

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