Development of the Structural Basis for Antimicrobial and Hemolytic Activities of Peptides Based on Gramicidin S and Design of Novel Analogs Using NMR Spectroscopy*

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The structures of 14-residue head-to-tail cyclic gramicidin S peptides have been investigated to develop the structural rationale for their antimicrobial and hemolytic profiles. The basis for these studies is GS14 (cyclo-(VKLKVdYPLKVdYP)), designed as an extension of the naturally occurring antimicrobial peptide. The structure of GS14 has been determined using NMR methods and was found to exist in a highly amphipathic antiparallel β-sheet conformation. Systematic enantiomeric substitutions within the framework of the GS14 peptide were found to decrease the amphipathicity of this molecule. These results indicated that there was a direct correlation between the high amphipathic character and potent hemolytic activity in the diasteromers, whereas an inverse correlation existed between amphipathicity and antimicrobial function. To define the structural consequences of changing the amphipathic nature of GS14 analogs to maximize antimicrobial activity and to minimize hemolysis, NMR structures were determined in water and the membrane-mimetic solvent trifluoroethanol. The structures show that these attributes are the result of induction of the β-sheet character in a membrane environment and the positioning of charged side chains on the hydrophobic face of the cyclic framework, thus decreasing the amphipathicity and directed hydrophobicity of these molecules. Implications for the design of more effective antimicrobials are discussed.

Research into the development of new classes of antibiotics to counteract bacterial resistance has been intensely pursued in recent years. The search for new molecules has led to the study of naturally occurring antimicrobial peptides as a foundation on which to target the lysis of microorganisms by mechanisms that do not aid in the proliferation of resistant strains (1, 2). Molecules based on the 10-residue head-to-tail cyclic peptide gramicidin S (3) have shown considerable promise as potential antibiotics (4). Although the precise mechanism of antimicrobial action is uncertain at present, evidence suggests that peptide accumulation in the bacterial membrane results in a permeability increase and loss of barrier function (2, 5, 6).

Since these molecules target the cell membrane, the development of resistance is unlikely, as this would require multiple mutations in the metabolic pathways leading to cell membrane synthesis and also necessitate structural rearrangements of the membrane itself. To define the mechanism of action of these peptides in greater detail, it is first essential to delineate the structural features of the molecules that are requisite for their functional behavior. An understanding of the structure-function relationship should not only allow the development of more efficacious molecules, but may also give an indication of how the membrane can be targeted in a more productive manner. The structure-function relationship is also essential for the purposes of determining the factors that specify the toxicity of the gramicidin S derivatives for higher eukaryotic cells and thus for decoupling the deleterious side effects of these compounds from their useful therapeutic benefit.

At present, a series of recently reported peptides based on the 14-residue gramicidin S derivative (GS14) show considerable potential for development of novel antimicrobial agents since they have high specificity as indicated by high antimicrobial activity and low hemolytic function (7). GS14 is a 14-residue head-to-tail cyclic molecule that possesses a poor therapeutically index (ratio of antimicrobial action to hemolytic activity). The NMR structure of GS14 has been previously determined and shown to exist as a completely amphipathic antiparallel β-sheet structure, i.e. with the 6 hydrophobic residues on one face of the β-sheet and the 4 hydrophilic basic residues on the opposite face (8, 9). Structure-function analysis of these analogs and of other types of antimicrobial peptides suggests that the requisite features for antimicrobial activity include a defined secondary structure in conjunction with amphipathic separation of hydrophobic residues on one face and basic residues on the opposing face (1, 2, 4, 10). Further studies have also shown that high amphipathicity, in addition to high hydrophobicity, contributes to elevated hemolytic activity, whereas an inverse correlation has been observed between these properties and antimicrobial action for a variety of antimicrobial peptides (9, 11–13).

A recent study in particular illustrated this for a series of diastereomeric peptides of GS14 in which each residue in the molecule was systematically replaced by its enantiomer (7). The results demonstrated that the RP-HPLC measure of amphipathicity was directly correlated with hemolytic activity and inversely correlated with antimicrobial activity.

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1 The abbreviations used are: RP-HPLC, reversed-phase high performance liquid chromatography; TFE, 2,2,2-trifluoroethanol; NOESY, nuclear Overhauser effect correlation spectroscopy; NOE, nuclear Overhauser effect.
The diastereomers possessing some of the lowest amphipathicities possessed the greatest specificity for microbes over erythrocytes as measured using a therapeutic index defined as the ratio of hemolytic activity to antimicrobial activity. These data, coupled with CD experiments, indicate that disruption of the \( \beta \)-sheet structure of the GS14 molecule by the incorporation of enantiomeric substitutions reduces the amphipathic character of the parent molecule and results in more desirable functional activity. Despite the understanding in terms of relative amphipathicity and the indication of structural perturbation by CD analysis, a precise conformational analysis of the diastereomeric analogs is not at present available. Structure determination using NMR is currently the only method by which the detailed conformational features of these peptides can be assessed and is essential for the development of a structure-function profile for this potential new class of antimicrobial therapeutic agents. This study therefore reports on the use of NMR techniques to study the solution structure of GS14 peptides to elucidate the structural changes induced by introduction of enantiomeric substitutions and thus to characterize at the molecular level the basis for the decreased amphipathicity of the potential therapeutic analogs. Furthermore, on the basis of the derived structural information, we have designed a novel analog that possesses similar desirable properties to the best GS14 diastereomer, with the exception that it exists in a preformed \( \beta \)-sheet structure.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis, Purification, and Cyclization—**GS14 analogs were synthesized using standard solid-phase peptide synthesis employing t-butyloxy carbonyl methodology. After cleavage from the resin, the peptides were purified using preparative RP-HPLC as reported previously (9). For the purposes of cyclization, peptides were produced with proline as the C-terminal residue to prevent racemization induced by the cyclization process. The homogeneity of the linear peptides was confirmed by analytical RP-HPLC and correct peptide masses were verified by electrospray mass spectrometry on a Fisons VG Quattro triple quadrupole mass spectrometer. Subsequent to purification, the linear side chain-protected peptides were cyclized, deprotected, and reprecipitated by preparative RP-HPLC. The cyclized peptides were shown to be homogeneous by analytical RP-HPLC and gave the expected primary ion molecular weights by mass spectrometry in addition to yielding appropriate amino acid analysis ratios.

**Analytical Reversed-phase Analysis of Peptides—**Peptides were analyzed by RP-HPLC on a Zorbax SB-C8 column (2.1 inner diameter) \( \times \) 150 mm particle size, 300-A pore size, Rockland Technologies (Wilmington, DE) using a Hewlett-Packard 1100 chromatograph at 70 °C with a linear solvent A/B gradient of 1% solvent B/min (where solvent A was 0.5% aqueous trifluoroacetic acid and solvent B was 0.5% trifluoroacetic acid in acetonitrile) at a flow rate of 0.25 ml/min.

**Measurement of Antimicrobial and Hemolytic Activities—**Antimicrobial and hemolytic activities were measured essentially as reported previously (9). For antimicrobial activity measurements, the peptide was dissolved in a volume of 500 \( \mu l \) of either \( H_2O \) or TFE/H\(_2\)O containing 10\% \( D_2O \) and 0.10 m\( k \) sodium 2,2-dimethyl-2-silapentane-5-sulfonate (internal standard), and the pH was adjusted to 5.3 using small aliquots of DCI or NaOD. The peptide concentrations were 4 and 8 \( \mu k \) for the two-dimensional homonuclear and heteronuclear NMR experiments, respectively.

**RESULTS**

**Rationale for Structural Analysis of GS14 Analogs**

The systematic replacement of each of the residues of GS14 by its enantiomeric counterpart revealed that a gradated profile exists for disruption of the \( \beta \)-sheet and the amphipathic nature compared with the parent molecule (7). From this series of diastereomers, GS14K4 was selected for structure determination since it displayed the highest efficacy in terms of maximizing antimicrobial activity and minimizing hemolytic action, i.e., it exhibited the highest therapeutic index. As shown in Table I, the creation of GS14K4 by the incorporation of an enantiomeric substitution at position 4 of the GS14 framework resulted in a significantly lower retention time on RP-HPLC compared with GS14 itself. Peptide interaction with reversed-phase matrices is mediated primarily through hydrophobic interactions. In the case of GS14, which exists as a highly amphipathic \( \beta \)-sheet compound, interaction occurs with the large amphipathic \( \beta \)-sheet and the amphipathic \( \alpha \)-helical part of the molecule. Since GS14 and GS14K4 have exactly the same residue composition and sequence and thus identical intrinsic hydrogen-bonding properties, any differences in their behavior on RP-HPLC are due to differences in their structure and, more specifically, differences in their amphipathicity. Thus, the decreased retention time of GS14K4 relative to GS14 is due to decreased amphipathicity of this analog. The decreased amphipathicity of GS14K4 relative to GS14 results in decreased hemolytic activity, as shown in Table...
I. Whereas GS14 is highly hemolytic at both 10 and 100 μg/ml, GS14K4 exhibits negligible hemolysis at these concentrations. As shown in Table II, the decreased amphipathicity of GS14K4 also results in increased antimicrobial activity against both Gram-positive and Gram-negative microorganisms. GS14 is essentially inactive against the Gram-negative microorganisms shown, with a minimum inhibitory concentration of 128 μg/ml, whereas GS14K4 exhibits moderate activity against these microorganisms, with a minimum inhibitory concentration on the order of 10-fold lower than that of GS14. Similarly, whereas GS14 exhibits moderate activity against the Gram-positive microorganisms shown in Table II, GS14K4 is significantly more active against these microorganisms.

The structural basis for the decreased amphipathicity of the GS14 diastereomers was not directly apparent from the CD studies of this series of peptides in water since most of the peptides exhibited similar CD spectra under aqueous conditions (7). However, in the corresponding TFE analyses, significant differences were observed with each analog. Of the diastereomeric series, next to GS14, GS14K4 possesses a similar β-sheet-like structure in water (8). These are illustrated in Fig. 2 for the aqueous model (8). These are illustrated in Fig. 2 for the aqueous environment, at least in part because of a limited number of experimental restraints; however, a major and minor family of structures are present. From observation of the conformational change, ensembles of solution structures were calculated for GS14K4 under aqueous conditions. From the data sets collected for the peptide in water, 102 NOE-SY distance restraints (13 non-equivalent NOEs of which 6 were cross-strand) and seven torsion angle restraints were obtained and used in X-PLOR simulated annealing calculations to generate a family of structures that satisfied the experimental data. From superposition of the structural ensemble, it is apparent that significant flexibility of the molecule exists in the aqueous environment, at least in part because of a limited number of experimental restraints; however, a major and minor family of structures are present. From observation of the major conformation, it is evident that under these conditions, GS14K4 partially exists in a β-sheet-like secondary structure as evidenced by the ϕ and ψ torsion angles that fall within the range for an antiparallel sheet. There is also evidence from the ensemble for proximity of cross-strand atoms, including the α-protons of residues 4 and 9 and residues 2 and 11, and for the expected cross-strand hydrogen bonds between Lys9 and Val15, Lys11 and Leu12, and Lys15 and Val17 (7), thus further indicating that this diastereomer exists in a β-sheet-like conformation. In addition, the two type II’ β-turns that are present in GS14 are also observed in a similar conformation in GS14K4 and therefore allow the two strands to be brought into proximity to form the antiparallel sheet. Comparison of the average structures for GS14 (8) and GS14K4 in water indicates that they adopt similar backbone conformations in this environment. The two peptides also adopt similar side chain confor-

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**TABLE I**

| Peptide     | Sequence | HPLC | MHC | Hemolytic activity
|-------------|----------|------|-----|---------------------|
|             |          | μg/ml| μg/ml | %
| GS14        | VKLVDYPLKVKLVDYP | 50.9 | 150 | 100 |
| GS14K4      | VKLVDYPLKVKLVDYP | 38.5 | 200 | 8 |
| GS14K3L4    | YPLKVKLYPLKVKL | 39.9 | ND  | 37 |

a Shown are the linear sequences of cyclic peptides. GS14K4 incorporates a d-amino acid enantiomeric substitution at position 4 of the GS14 sequence, and GS14K3L4 incorporates an interchange of residues 3 and 4 of GS14 to Lys-Leu.

b Retention time on reversed-phase HPLC.

c Hemolytic activity for 100% lysis (data from Ref. 7).

d Percent hemolysis of human red blood cells at the indicated peptide concentration.

**TABLE II**

| Peptide     | Minimal inhibitory concentration | Gram-positive | Gram-negative |
|-------------|---------------------------------|---------------|---------------|
|             | µg/ml | E. faecalis | S. aureus | S. epidermidis | E. coli | K. pneumonia |
| GS14        | 128   | 32   | 32    | 128   | 128   |
| GS14K4      | 16    | 4    | 4     | 8     | 8     |
| GS14K3L4    | 128   | 4    | 4     | 8     | 8     |

a Peptide concentration required to completely inhibit growth.

b Retention time on reversed-phase HPLC.

c Hemolytic activity for 100% lysis (data from Ref. 7).

d Percent hemolysis of human red blood cells at the indicated peptide concentration.

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**NMR Spectroscopy and Structure Determination of GS14K4**

Data Analysis under Aqueous Conditions—A complete assignment of most proton resonances of GS14K4 was made in H2O and TFE using standard two-dimensional experiments, and an initial examination of amide and α-proton shifts was used on a comparative basis as an indication of the structural differences between this diastereomer and the GS14 parent molecule (8). These are illustrated in Fig. 2 for the aqueous environment, at least in part because of a limited number of experimental restraints; however, a major and minor family of structures are present. From observation of the major conformation, it is evident that under these conditions, GS14K4 partially exists in a β-sheet-like secondary structure as evidenced by the ϕ and ψ torsion angles that fall within the range for an antiparallel sheet. There is also evidence from the ensemble for proximity of cross-strand atoms, including the α-protons of residues 4 and 9 and residues 2 and 11, and for the expected cross-strand hydrogen bonds between Lys9 and Val15, Lys11 and Leu12, and Lys15 and Val17 (7), thus further indicating that this diastereomer exists in a β-sheet-like conformation. In addition, the two type II’ β-turns that are present in GS14 are also observed in a similar conformation in GS14K4 and therefore allow the two strands to be brought into proximity to form the antiparallel sheet. Comparison of the average structures for GS14 (8) and GS14K4 in water indicates that they adopt similar backbone conformations in this environment. The two peptides also adopt similar side chain confor-
motions, with the exception of Lys⁴ in GS14K4, which is no longer on the hydrophilic face of the sheet, but is now placed on the opposite face along with the 6 hydrophobic residues. The presence of the d-amino acid on this face confirms previous suggestions and gives a strong indication of the reduced amphipathicity of this analog compared with GS14. The significant changes in the α-proton chemical shifts between the two analogs for lysines 9 and 11 (Fig. 2) are most probably the consequence of movements in the backbone conformation required to accommodate the interchange of lysine 4 from the hydrophilic face to the hydrophobic side of the molecule.

Data Analysis in Aqueous Trifluoroethanol—To examine the structure of GS14K4 in a structure-promoting solvent and also in a membrane-mimicking environment, a series of NMR spectra were acquired for this analog in varying concentrations of TFE/H₂O. As shown in Fig. 3, which depicts the amide proton region of the one-dimensional spectra at the TFE concentrations indicated, addition of TFE and increasing its concentration resulted in a significant increase in the dispersion of the amide proton chemical shifts. This result suggests the induction of β-sheet secondary structure, and further evidence is provided by examination of the α-proton resonance chemical shifts, which underwent dramatic downfield shifts from 0 to 50% TFE at the expected positions for the close contact of the α-protons across the strands of the β-sheet. Lysines 2, 4, 8, 9, and 11 have Δδ values between water and TFE environments of 0.51, 0.71, 0.58, and 0.43 ppm, respectively, in addition to the smaller but significant shifts for the other residues involved in the two β-strands. Interpretation of the NOESY cross-peaks for GS14K4 in 30% TFE reveals that there is a substantial increase over the data acquired in water in the number and intensity of cross-strand NOEs corresponding to those expected for the β-sheet structure, as shown in Fig. 4. The evident inducement of secondary structure with the addition of TFE warranted further investigation, and to this end, structure calculations were performed using the 194 NOE-derived distances (including 31 non-sequential NOEs of which 23 were cross-strand) and eight coupling constant-derived dihedral restraints for the data set collected at 30% TFE/water. After refinement of the solution ensemble, a major family of structures was identified and demonstrated to adopt a similar but better defined structure compared with that observed under aqueous conditions. The backbone exhibits high definition with a root mean square deviation of 0.61 ± 0.10 Å; however, it is quite flexible at residues 4 and 5 due to the absence of a dihedral restraint for the d-lysine. The backbone dihedrals all fall within the range of values expected for an antiparallel β-sheet, with the exception of the turn residues and Lys⁴. The φ and ψ values for Lys⁴ are shifted by 180° relative to the other strand residues, which is an expected result for the d-amino acid configuration. The backbone dihedral angles for the structure generated for GS14K4 in 30% TFE/H₂O are shown in Table III.

Examination of the side chain arrangement of the ensemble
of TFE structures shows a similar presentation of hydrophobic and hydrophilic residues as that observed in the water structural family. As shown in Fig. 5, the \(d\)-lysine at position 4 projects onto the hydrophobic face of the \(\beta\)-sheet, whereas the other 3 lysine residues occupy the same positions on the hydrophilic face as observed in GS14. The orientation of lysine 4 on the same face as the valines and leucines gives an obvious structural rationale for the reduced amphipathicity and directed hydrophobicity of this diastereomer relative to GS14. In addition, the side chain of Val5 is rotated away from the hydrophobic face to accommodate the \(d\)-lysine projecting onto this surface. This is illustrated in Fig. 6, where the Connolly surface diagrams of the average NMR structures of GS14 and GS14K4 are presented. From this molecular surface representation of GS14K4, it is apparent that the surface of Val5 is no longer contiguous with the hydrophobic face and that the side chain of Lys4 projects onto this face. The rotation of the Val side chain away from the hydrophobic face gives further insight into the reduction in the amphipathicity and directed hydrophobicity of this analog.

**Design and NMR Analysis of GS14K3L4**

Based on the observation from the diastereomeric GS14K4 peptide that placement of the hydrophilic lysine on the hydrophobic face of the \(\beta\)-sheet dramatically increases the specificity for the bacterial membrane, the design of an analog with a similar relative positioning of the side chains was undertaken. We postulated that interchange of the GS14 sequence at positions 3 and 4, i.e. from Leu-Lys to Lys-Leu, would result in the placement of a lysine on the hydrophobic face and a leucine on the hydrophilic face of the designed molecule, GS14K3L4. How-
ever, unlike GS14K4, in which β-sheet structure was disrupted, GS14K3L4 was predicted to retain β-sheet structure. Preliminary structural characterization by CD spectroscopy indicates that GS14K3L4 does exist in a β-sheet conformation similar to GS14 under aqueous conditions (Fig. 1A) and, furthermore, that this structure is stabilized in the presence of TFE (Fig. 1B).

Assignment of amide and α-proton resonances along with 3JHNHα coupling constant information confirmed that GS14K3L4 indeed exists in a β-sheet conformation. NMR data collected in 30% TFE indicate that the β-sheet structure is further inducible as observed from the substantial downfield enhancement of the α-proton resonances. Structure calculations performed on this analog using a set of 141 NOE distance restraints (including 22 non-sequential NOEs of which 10 were cross-strand) confirmed the predicted arrangement of the side chains on the hydrophobic and hydrophilic faces, respectively. The average structure for GS14K3L4 depicted as a Connolly surface is shown at the bottom of Fig. 6 and illustrates the similar positioning of the lysine on the hydrophobic face of the molecule as seen in GS14K4. As shown in Table I, the retention time of GS14K3L4 on RP-HPLC is similar to, albeit slightly greater than, that of GS14K4. This indicates that the two peptides have similar amphipathicities since both peptides have the same residue composition. Similar to GS14K4, the hemolytic activity of GS14K3L4 is greatly reduced relative to GS14, although GS14K4 is slightly more hemolytic than GS14K4 as evidenced by the greater hemolysis at 100 μg/ml (Table I). The increased hemolytic activity relative to GS14K4 is likely due to the somewhat increased amphipathicity, as is evident by the slightly higher retention time on RP-HPLC. As shown in Table II, the antimicrobial properties of GS14K3L4 are essentially identical to those of GS14K4 and are substantially greater than those of GS14 for the Gram-positive and Gram-negative microorganisms evaluated. These findings again confirm the relationship between amphipathicity and activity and thus confirm the structural basis for the design of this analog.

As a whole, the NMR evidence collected from the GS14 analogs in aqueous and structure-promoting environments gives considerable insight into the conformational preferences and adaptability of these antimicrobial peptides. The implications of the structural data acquired are further discussed, and the basis for antimicrobial versus hemolytic activities is envisaged.

**DISCUSSION**

**Structural Basis for Decrease in Amphipathicity and Directed Hydrophobicity**—The analysis of NMR and CD data generated for GS14 analogs gives considerable insight into the structural basis for the antimicrobial and hemolytic activities of these molecules. The decreased amphipathicity of GS14K4 is undoubtedly due to the placement of the hydrophilic lysine side chain on the hydrophobic face of the β-sheet. Replacement of the l-lysine in the GS14 parent molecule with its d-counterpart results in rotation of the backbone bond angles to accommodate the steric interaction of the side chain with the opposite strand of the β-sheet. Placement of the lysine side chain on this face results in the φ and ψ torsion angles for a d-amino acid in a β-sheet being fulfilled and thus explains why the secondary structure is maintained in this analog. The reduction in the amphipathic character of GS14 through the placement of the hydrophilic residue on the hydrophobic face in conjunction with the rotation of the side chain of valine 5 away from this surface results in this analog undergoing a considerable increase in antimicrobial activity. This has been previously suggested to be the consequence of decreased high affinity binding to outer membrane components, which in turn facilitates penetration to and accumulation at the inner membrane and propagation of the effects leading to subsequent lysis of the bacterial cell membrane (7). This hypothesis is further corroborated by the NMR conformational analysis of the GS14K3L4 analog, which was designed based on the prediction that the exchange of positions 3 and 4 of GS14 would result in the placement of a lysine on the hydrophobic face in a manner analogous to GS14K4. This molecule exhibited a biological profile similar to that of the GS14K4 diastereomer in terms of antimicrobial and hemolytic activities (Tables I and II) and was demonstrated by CD and NMR studies to adopt the predicted conformation (Figs. 1 and 6). In an analogous manner to GS14K4, the disruption of the directed hydrophobicity through the placement of the charged residue on the hydrophobic binding face likely also results in diminution of the interaction with bacterial outer membranes.

With respect to hemolytic activity, it is presumed that the presentation of a large hydrophobic face as a consequence of high amphipathicity gives the impetus for lysis of eukaryotic membranes. This is supported by the observations that both increased hydrophobicity and amphipathicity contribute to increased hemolytic activity in either cyclic peptides (7, 9, 24) or linear unconstrained antimicrobial peptides (11–13). The placement of the lysine on the hydrophobic face of GS14K4 consequently reduces the directed hydrophobicity of this molecule and therefore diminishes the ability of the peptide to interact with human erythrocytes. This occurs since the exterior of these cells tends to be less ionic in nature, and therefore, greater selectivity for the microbial membranes is obtained. Since GS14 exhibits elevated hemolytic properties relative to GS14K4 and GS14K3L4, it is apparent that the presentation of a large hydrophobic surface, resulting from the amphipathic β-sheet structure, to the eukaryotic membrane invokes cell lysis. Disruption of this large hydrophobic face through the incorporation of d-lysine at position 4 in the case of GS14K4 or through the interchange of a leucine with a lysine as in GS14K3L4 leads to reduced amphipathicity, which results in reduced hemolytic activity. The decreased hemolytic activity of these analogs in conjunction with their increased antimicrobial activity results in greatly increased specificity of these peptides as antimicrobial agents.

**NMR-aided Characterization of GS14 CD Spectra and the Consequence of Trifluoroethanol β-Sheet Induction in GS14 Analogs**—As CD spectra for cyclic peptides are often difficult to interpret, NMR analysis and structure determination of these peptides greatly assist in the characterization of structural variations observed from CD data. Examination of the CD spectra for the various GS14 diastereomers in aqueous solution
indicates that, in most cases, the incorporation of enantiomeric substitutions within the framework of GS14 results in disruption of the β-sheet structure (7). Confirmation of this perturbation is obtained from the NMR parameters in water, which indicate reduced β-sheet content. Specifically, calculation of the chemical shift index demonstrates that the trend in α-proton shifts is toward random coil values, and in addition, the three-dimensional ensemble generated for GS14K4 under aqueous conditions displays β-sheet structures, but they exist in multiple conformations. In addition, since the antiparallel β-sheet was demonstrated to be highly inducible for GS14K4 in 30% TFE through examination of a variety of NMR parameters and determination of the three-dimensional structure, the unusual CD spectrum obtained for this analog can be interpreted. The spectrum of GS14K4 in TFE exhibits a β-sheet-like curve; however, the minimum is shifted from 208 to ~200 nm (Fig. 1, A and B). Due to the identification of the presence of the β-structure in the GS14K4 analog, the blue-shifted ellipticity can be attributed to the differential contribution to the CD spectrum of the d-lysine relative to the l-lysine in the parent GS14 molecule as well as the increased strain on the backbone due to this substitution.

The inducement and stabilization of the antiparallel β-sheet in these analogs suggest that to effect penetration and lysis of bacterial membranes and to prevent association with the eukaryotic membrane, the hydrophobic binding domain of the partially amphipathic molecule must exist; however, it must be concurrent with the location of a hydrophilic residue within this interface. This can be concluded since the β-sheet structure only partially exists in aqueous solution with GS14K4 and is propagated in TFE, resulting in more rigid presentation of the side chains on the hydrophobic side of the peptide. On the other hand, GS14K3L4 appears to have a considerably more stable β-sheet structure under aqueous conditions as evidenced by a CD spectrum similar to that of GS14. As shown by both CD and NMR spectroscopies, the structure is stabilized considerably in TFE, thus indicating that a more ordered presentation of the side chains on the hydrophobic face of the peptide. In interpreting the hemolytic tendencies of the GS14 analogs, the induction of β-sheet structure and the resulting ordered presentation of the side chains, including basic side chains on the hydrophobic face of the molecule, result in a significant decrease in the affinity of these peptides for the erythrocyte membrane compared with the parent GS14 molecule. Since the secondary structure induced by TFE results in stabilization of the β-sheet and the side chain presentation of the hydrophobic face, it is probable that this solvent produces structures representative of those occurring in the environ-

**FIG. 5.** Ensemble of NMR solution structures calculated for GS14K4 with the program X-PLOR using restraints obtained from the NOESY and coupling constant information acquired in 30% TFE. Two views of the molecule are shown, with the peptide backbone colored black, hydrophobic residues in green, and the four lysines in blue. From both views of the molecule, it can be seen that the d-lysine at position 4 projects onto the hydrophobic face of the β-sheet.

**FIG. 6.** Connolly surface representation of the average structures of GS14K4 (top), GS14 (center), and GS14K3L4 (bottom) calculated from the NMR ensemble. From this view of GS14K4, the occupation of the d-lysine at position 4 on the hydrophobic face can be clearly seen. For GS14K3L4, the lysine at position 3 that was interchanged with the leucine is positioned on the hydrophobic face of this peptide. The peptide backbone is colored black; hydrophobic residues are in green; and the four lysines are in blue.
ment of the biological membrane. Further experiments will be needed to confirm this hypothesis, and solution- and solid-state NMR experiments in the presence of membranes may prove valuable in the elucidation of the complete mechanism of antimicrobial action of gramicidin S compounds.

In summary, through the use of NMR-derived structural information, we have shown that the structural basis for the emergence of biologically desirable activities (low hemolytic activity coupled with high antimicrobial activity) is the decreased amphipathicity of certain GS14 analogs. The decrease in GS14 amphipathicity was achieved by two different methods. In one approach, as exemplified by GS14K4, the incorporation of an enantiomeric substitution within the framework of the highly amphipathic β-sheet peptide GS14 resulted in destabilization of the β-sheet structure and positioning of a basic residue on the hydrophobic face of the molecule, thereby reducing amphipathicity. In a second approach, based on the detailed knowledge of the three-dimensional structure of GS14K4, interconversion of 2 residues in GS14 resulted in the analog GS14K3L4, which possesses similar amphipathicity compared with GS14K4, but retains a stable β-sheet conformation. Regardless of the approach taken, both analogs possess a similar amphipathicity and structure in hydrophobic environments, which lead to essentially identical desirable biological properties.

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