Effects of Phenylalanine Substitutions in Gramicidin A on the Kinetics of Channel Formation in Vesicles and Channel Structure in SDS Micelles

J. B. Jordan, P. L. Easton, and J. F. Hinton
Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas

ABSTRACT The common occurrence of Trp residues at the aqueous-lipid interface region of transmembrane channels is thought to be indicative of its importance for insertion and stabilization of the channel in membranes. To further investigate the effects of Trp→Phe substitution on the structure and function of the gramicidin channel, four analogs of gramicidin A have been synthesized in which the tryptophan residues at positions 9, 11, 13, and 15 are sequentially replaced with phenylalanine. The three-dimensional structure of each viable analog has been determined using a combination of two-dimensional NMR techniques and distance geometry-simulated annealing structure calculations. These phenylalanine analogs adopt a homodimer motif, consisting of two $\beta^{6.3}$ helices joined by six hydrogen bonds at their NH_2-termini. The replacement of the tryptophan residues does not have a significant effect on the backbone structure of the channels when compared to native gramicidin A, and only small effects are seen on side-chain conformations. Single-channel conductance measurements have shown that the conductance and lifetime of the channels are significantly affected by the replacement of the tryptophan residues (Wallace, 2000; Becker et al., 1991). The variation in conductance appears to be caused by the sequential removal of a tryptophan dipole, thereby removing the ion-dipole interaction at the channel entrance and at the ion binding site. Channel lifetime variations appear to be related to changing side chain-lipid interactions. This is supported by data relating to transport and incorporation kinetics.

INTRODUCTION

Amphipathic residues such as tryptophan (Trp) are believed to play a critical role in the structure and function of integral membrane proteins (Wallace, 2000; Killian et al., 1985; Becker et al., 1991; Schiffer et al., 1992; Andersen et al., 1998). It is strongly believed that the common occurrence of Trp residues at the aqueous-lipid interface region of transmembrane channels is indicative of its importance for insertion and stabilization of the channel in the membrane (Killian et al., 1985; Henderson et al., 1990; Schiffer et al., 1992; Hu et al., 1993; Wallace and Janes, 1999). Trp possesses several properties that are thought to make it ideal for this purpose. Among these are Trp's amphipathic characteristics and the ability to form hydrogen bonds. The Trp side chain also possesses a dipole moment of ≈2.08 D, a property believed to play a large role in ion binding and transport functions of the gramicidin ion channel (Hu, et al., 1993; Sham et al., 2003). For these reasons, Trp residues have been the target of many recent studies (Becker et al., 1991; Hu et al., 1993; Hu and Cross, 1995; Yau et al., 1998; Andersen et al., 1998; Cotten et al., 1999; Sham et al., 2003). Investigated here is the role Trp residues play in determining the structural and functional properties of the model ion channel system gramicidin A (gA).

Ion channels formed by gA, gramicidin B (gB, with Phe at position 11 instead of Trp), and gramicidin C (gC, with Tyr at position 11 instead of Trp) adopt single-stranded, right-handed, $\beta^{6.3}$ helical homodimers, with both monomers being joined by hydrogen bonds at their NH_2-termini (Urry et al., 1971; Arseniev et al., 1985; Russell et al., 1986; Townsley et al., 2001). Small structural differences observed in the three analogs have been attributed to variations in their respective amino acid sequences (Townsley et al., 2001). However, large differences have been observed in the functional characteristics of gA, gB, and gC (Table 1). It is believed that variations in the observed single-channel conductance and lifetimes of these analogs have much to do with the physiochemical properties of residue 11 in a given environment (Sawyer et al., 1990; Townsley et al., 2001). With replacement of Trp_{11} (dipole moment ≈2.08 D) in gA by Phe (dipole moment ≈0.0 D) in gB, single-channel conductance values decrease from 15.0 pS to 8.7 pS; however, substitution with Tyr (dipole moment ≈1.54 D), as in gC, causes a much smaller decrease, from 15.0 pS to 13.3 pS (Table 1) (Townsley et al., 2001, and references therein). It is thought that the variation in dipole moments affect long range ion-dipole interactions critical for ion conduction. In addition, variation of the amino acid residue at position 11 (e.g., Trp, Phe, or Tyr) also induces changes in the average lifetime of channel species (Mazet et al., 1984; Becker et al., 1991; Williams et al., 1992; Andersen et al., 1998). The

Submitted June 14, 2004, and accepted for publication October 15, 2004.

Address reprint requests to J. F. Hinton, Dept. of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701. Tel.: 501-575-5143; Fax: 501-575-4049; E-mail: jhinton@uark.edu.

Abbreviations used: gA, gramicidin A; Phe_{11}-gA, Phe_{11}-gramicidin A; d_2-TFE, deuterated trifluoroethanol (CF_3CD_2OH); d_{25}-SDS, deuterated sodium dodecyl sulfate; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; DGSA, distance geometry/simulated annealing; LUV, large unilamellar vesicle; SDS, sodium dodecyl sulfate; RMSD, root mean-square deviation.

0006-3495/05/01/224/11 $2.00

doi: 10.1529/biophysj.104.047456

TABLE 1 Single-channel conductances and average lifetimes

Analog	Conductance* (pS)	Lifetime (ms)
Gramicidin A	15.0 ± 0.3	840
Gramicidin B†	8.7 ± 0.3	2300
Gramicidin C	13.3 ± 0.3	950
Phe_{15}-gA	10.9 ± 0.1	790
Phe_{13}-gA	11.2 ± 0.1	800
Phe_{9}-gA	6.0 ± 0.1	1000

Single-channel conductances and average durations are given as measured in DPhPC/*n*-decane bilayers under conditions of 1.0 M NaCl, 200 mV, and 25 ± 1°C.
*Conductance values are given as mean ± SD.
†Phe_{11}-gA.

average single-channel lifetime is a measure of the gramicidin dimer dissociation rate constant (Becker et al., 1991), and provides a measure of channel stability in a bilayer mimetic. It is believed that the influence of Trp on channel lifetime is a complicated function of both enthalpic and entropic contributions, which are directly related to the interactions between the indole-NH of the Trp side chains and the interfacial H_2O, headgroups, and phospholipid backbones (Sawyer et al., 1990; Becker et al., 1991; Cotten et al., 1999).

The literature contains many single-channel conductance studies in which Trp residues of gramicidin have been replaced by less polar or more polar side chains (Tredgold and Hole, 1976; Mazet et al., 1984; Etchebest and Pullman, 1985; Koeppe et al., 1990, 1998; Becker et al., 1991; Andersen et al., 1998; Busath et al., 1998; Dorigo et al., 1999; Cotten et al., 1999; Phillips et al., 1999; Thompson et al., 2001; Anderson et al., 2001; Cole et al., 2002; Sham et al., 2003), and where the hydrogen bonding abilities of Trp residues have been removed by N-methylation (Sun et al., 2002). In addition, solid-state NMR experiments show that Trp side-chain dipoles in gA channels are oriented such that they can lower the barrier for ion movement through the channel (Hu et al., 1993, 1995; Koeppe et al., 1994). All of these results suggest that electrostatic interactions between transport ions and the dipoles of Trp side chains serve in determining the functional ability of the channel, a property that can be observed by single-channel conductance measurements. To aid in understanding how modulation of the Trp residues can affect ion conduction, one can picture transport in these channels as a three-step process (Durkin et al., 1990). These steps include 1), movement of the ion to the channel entrance; 2), binding and translocation of the ion through the channel; and 3), exit of the ion from the channel. From the known structure of the gramicidin channel, it can be postulated that variations in ion-dipole interactions near the C-terminus could affect entrance and exit of the ion from the channel. As for ion translocation, the movement of the transport ion through the channel must involve binding and release of the ion at the ion binding site. Differences in binding constants for cations have been related to variations in side-chain polarity at the ion binding site of the channel (Hinton, 1999). Most recently, the Na^+ binding site in gramicidin has been estimated by molecular dynamics and solid-state NMR data to exist ~9.2 Å from the channel center (Woolf and Roux, 1994). These experiments suggest that the Na^+ ion makes contact with the carbonyl oxygen atoms of Leu_{10} and Trp_{15} and that the main channel ligand is provided by the carbonyl group of the amide bond between the Leu_{10} and Trp_{11} residues. With this in mind, it is not surprising to find that changes in ion-dipole interactions near these residues are very important in modulating the transport of ions through the channel. This evidence supports the idea that variations in ion-dipole interactions nearer the channel interior (i.e., positions 9–11) would be more likely to affect ion binding and translocation.

It has been found that single-channel properties of gramicidin analogs depend highly on the position of substitution (i.e., 9, 11, 13, or 15) and the orientation and magnitude of side-chain dipole moments (Becker et al., 1991; Cotten et al., 1999; Sham et al., 2003). To assess functional differences caused by Trp substitutions and assign them to any structural changes, various analogs must be synthesized in which the dipole moments at these Trp positions are altered, and their three-dimensional structures must be determined.

Recently, it was reported by this laboratory that replacement of Trp_{15} in gramicidin A with Gly resulted in a significant decrease in single-channel conductance and lifetime, though only small differences in structural properties were observed (Sham et al., 2003). This replacement resulted in removal of the dipole of residue 15 and a significant change in side-chain structure at the substitution site (Trp → Gly, a nonconservative replacement). The main interest here is to investigate whether a similar impairment of channel function will occur when using a conservative substitution that more closely resembles that of the native Trp, but lacks a side-chain dipole moment (Trp → Phe).

Results presented here are a continuation of a study in which phenylalanine (Phe) analogs of gramicidin A were synthesized and their structure determined and properties measured. In an attempt to correlate previously determined conductance and channel lifetime data with structural changes due to substitutions of Trp residues, the three-dimensional structures have been determined for gA analogs in which Trps at positions 11, 13, and 15 are replaced systematically by Phe. The structures were determined using the combination of two-dimensional NMR spectroscopy and molecular modeling.

To determine the effect of the Phe substitution on channel formation, the kinetic activation enthalpy and entropy for the insertion of Phe analogs into vesicles concomitant to channel formation have been determined using a ^{23}Na NMR technique developed in this laboratory (Easton et al., 1990).

MATERIALS AND METHODS

Gramicidin analogs were prepared synthetically using solid-phase fmoc synthesis on an Applied Biosystems 431A Peptide Synthesizer (Foster City,

CA). The amino acid sequence of each analog was confirmed with an Applied Biosystems 473A Protein Sequencer (Foster City, CA). All analogs were doubly purified using high-performance liquid chromatography. The purity (>98%) of each analog was determined using electrospray ionization mass spectrometry (Mass Consortium, San Diego, CA, and the University of Arkansas Mass Spectrometry Center).

Nondeuterated sodium dodecyl sulfate (SDS) was obtained from PGC Scientifics (Gaithersburg, MD). d_{25}-SDS, D_2O, and d_2-TFE were obtained from Cambridge Isotope Laboratories (Cambridge, MA). The d_{25}-SDS was recrystallized twice from 95% ethanol and stored under vacuum for 24 h before sample preparation. A 100-mM phosphate buffer solution, pH 6.5, was purchased from PGC Scientifics.

Phosphatidylcholine and phosphatidylglycerol used in the transport and incorporation studies were purchased from Sigma Chemicals (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL), respectively.

Sample preparation for structure determination and channel formation kinetics

Gramicidin analogs, incorporated into the SDS micelles, were first screened for structural homogeneity using methods previously published (Hinton et al., 2002; Hinton et al., 1997; Hinton and Washburn, 1995). Using nondeuterated SDS, the indole-NH NMR signals were observed for each analog. The existence of a single helical species was confirmed by the presence of the correct number of signals (9–11 ppm; four for gA, three for the Phe analogs). The presence of more than the expected number of signals indicated the existence of multiple helical forms. If samples were found to be structurally heterogeneous, the SDS concentration was increased, and the samples screened again. Once a suitable peptide:SDS ratio was found where only one form existed, the analogs were incorporated into deuterated SDS micelles at an identical concentration. Phe_{15}- and Phe_{11}-gA produced single channel species at the initial 1:50 peptide/SDS ratio (5 mM peptide/250 mM SDS), and Phe_{13}-gA required a 1:150 ratio (5 mM peptide/750 mM SDS). Phe_9-gA failed to produce a single species even at very high (~800 mM) SDS concentrations.

Solutions used for ^{1}H 2D-NMR experiments were prepared in the following manner. A 50-mM solution of the analog dissolved in d_2-TFE was combined with 275 mM aqueous d_{25}-SDS solution (90% pH 6.5 buffer/10% D_2O, v/v). This yielded a final concentration of ~5 mM peptide and 250 mM d_{25}-SDS in 80% pH 6.5 buffer/10% D_2O/10% d_2-TFE. (825 mM SDS solution was used for the Phe_{13}-gA analog, yielding a final concentration of 5 mM peptide/750 mM SDS). Samples were then sonicated at low power for 5 min to aid in the incorporation of the peptide into the micelles.

The procedures for forming LUVs, incorporating the gramicidin A analogs into the vesicles, and placing Na^+ ions on the inside and outside of the vesicles have been presented in detail in the literature (Hinton et al., 1993).

NMR experiments for structure determination

NMR experiments were performed using a Varian VXR 500S and Bruker Avance 500 MHz NMR spectrometer. Spectra were recorded at 55°C in phase-sensitive mode using the States-TPPI method. Solvent suppression was achieved using transmitter presaturation. Resonance assignments for all protons were made using the DQF-COSY (Piantini et al., 1982), TOCSY (Braunschweiler and Ernst, 1983), and NOESY (Jeener et al., 1979) experiments.

The DQF-COSY experiment was acquired in 512 t_1 increments with 8192 data points. One hundred twenty-eight transients were used per t_1 increment. The TOCSY experiment was acquired in 256 t_1 increments using 64 transients/increment, 4096 data points, and a mixing time of 75 ms with the MLEV-17 mixing sequence. The NOESY experiment was acquired in 512 t_1 increments using 128 transients/increment and 8192 data points. The NOESY experiments utilized a 70-ms mixing time, which was found to be within the linear range of the NOE buildup. All NMR data were processed using the FELIX software available from Accelrys (San Diego, CA).

Structure determination

Calculation strategies used to determine the three-dimensional structures utilized experimentally determined NOE and hydrogen bonding restraints in coordination with the software package CNS/ARIA (Brunger et al., 1998; Linge et al., 2003) and a home-built Linux cluster. A summary of restraint information used in the calculations is provided in Table 5. DGSA protocols were used with torsion angle dynamics as a calculation method. The structure of gramicidin A that is currently published in the Protein Data Bank (1JNO) was calculated using the program DSPACE 4.0 (Hare Research, Woodinville, WA). To be consistent, the structure of native gramicidin A used in this article was redetermined using newly acquired NMR data and the programs CNS and ARIA (Brunger et al., 1998; Linge et al., 2003). Structures of gramicidin A calculated by the two methods were found to be very similar (RMSD <1.0 Å), with small differences existing primarily in the nonaromatic side chains. For calculation of the homodimer structures, each channel was first modeled as a monomer. Noncrystallographic symmetry and symmetry restraints were then used with modified DGSA protocols to include these symmetry restraints in the calculation of the symmetric dimer.

NMR experiments for kinetics of channel formation

The kinetics of thermal incorporation and channel formation of the Phe analogs of gramicidin A into LUVs was studied using a ^{23}Na NMR spectroscopy method developed in this laboratory in which one can observe Na^+ ions in the aqueous pools on the inside and outside of the LUVs (Easton et al., 1990). This technique is based upon a modification of earlier experiments in which changes in the ^{23}Na NMR linewidth of the Na^+ ions on the inside aqueous environment of the LUVs (as opposed to those Na^+ ions on the outside of the LUVs) was used to obtain the rate constant for the transport process (Buster et al., 1988). The incorporation of ion transporting gramicidin channels into vesicle membranes under ionic equilibrium conditions requires a thermal incubation period. Line broadening of the ^{23}Na NMR signal of the inside peak occurs as the number of channels in the membrane increases with the time of thermal incubation. This line broadening as a function of time can then be used to obtain the rate constant for channel incorporation and formation in the LUV membrane at the temperature of incubation. Therefore, the kinetic activation enthalpy ($\Delta H^{\pm}$) and entropy ($\Delta S^{\pm}$) for this process can be determined by obtaining the rate constant as a function of the incubation temperature. The values of ($\Delta H^{\pm}$) and ($\Delta S^{\pm}$) of incorporation for gramicidin A and the four Phe analogs are shown in Table 3.

RESULTS AND DISCUSSION

Three-dimensional structures of native gA and its Phe_{11}, Phe_{13}, and Phe_{15}- analogs have been determined using NMR methods and DGSA. Previous research has shown that increasing the peptide/SDS ratio can induce substituted gramicidins to adopt a single helical species, enabling determination of their structures by solution NMR (Hinton and Washburn, 1995; Hinton et al., 1997). Even at the highest useable SDS concentration (~800 mM), the NOESY spectrum of Phe_9-gA was so complex that individual species could not be defined. For this reason, Phe_9-gA has been excluded from the structure calculations. Presented here is

a comparison of the three-dimensional structures of Phe_{11}-, Phe_{13}-, and Phe_{15}-gA with native gA. In addition, the functional properties (Hinton et al., 1988a; Becker et al., 1991), and kinetics of ion transport (Hinton et al., 1993) and incorporation (Easton, 1989) of Phe_9-, Phe_{11}-, Phe_{13}-, and Phe_{15}-gA are compared to those of native gA. Results indicate that a delicate balance exists between structural changes, dipole alteration, side chain-lipid interactions, and effects on the functional properties of gA channels.

The backbone-NH region of the NOESY spectra for gA and its Phe_{11}, Phe_{13}, and Phe_{15} analogs are shown in Figs. 1 and 2. Minimized average structures of gA and each of the Phe analogs are shown in Fig. 3 and are accompanied by

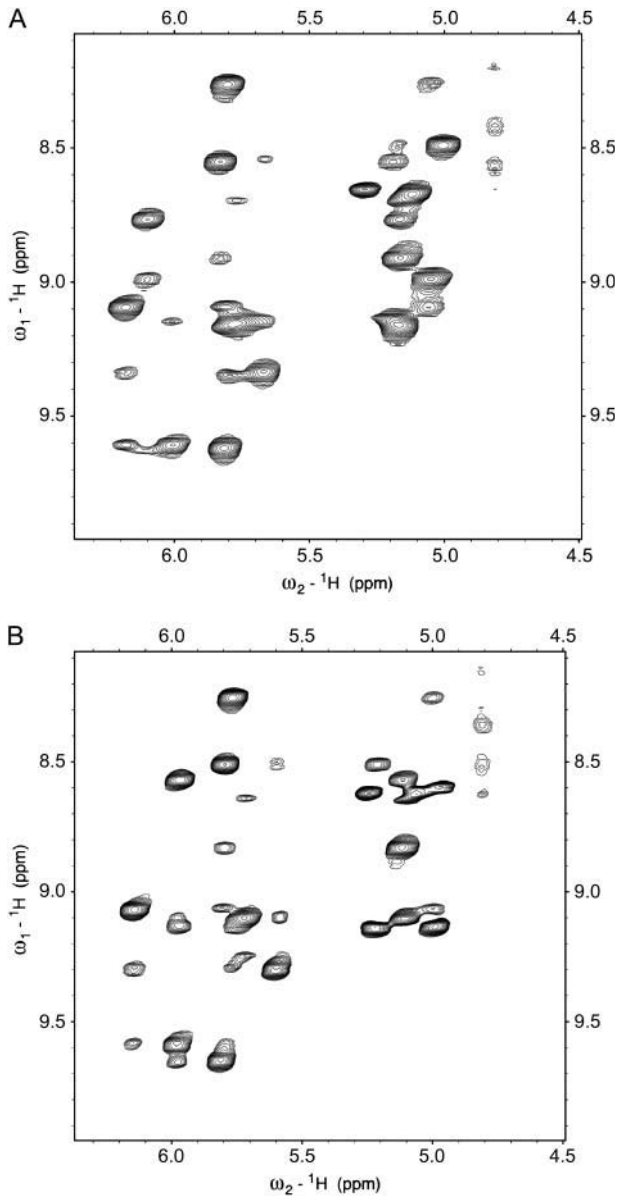

FIGURE 1 Backbone indole-NH region of the NOESY spectra of (*A*) native gA and (*B*) Phe_{11}-gA.

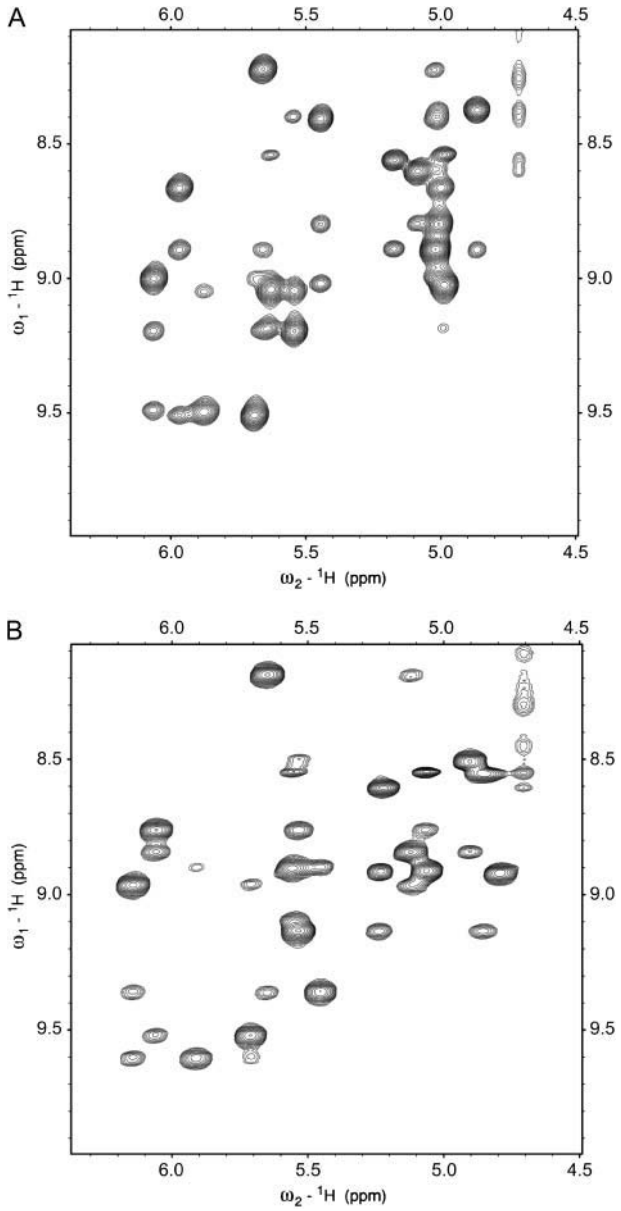

FIGURE 2 Backbone indole-NH region of the NOESY spectra of (*A*) Phe_{13}-gA and (*B*) Phe_{15}-gA.

superpositions of the 10 best structures obtained for each analog in Figs. 4 and 5. Fig. 6, *A–C*, shows transverse overlay plots of each analog with native gramicidin A. To increase clarity for comparison, the formyl and ethanolamine capping groups are excluded. Ethanolamine is known to exhibit extensive conformational flexibility (Townsley, 2000), and formyl groups, residing at the NH_2-terminal interface, are far removed from the areas of interest and exhibit no structural effects from the substitutions. Summarized in Tables 1 and 2 are data previously obtained in single-channel conductance studies and the kinetic parameters for ion transport for each analog (Becker et al., 1991; Hinton

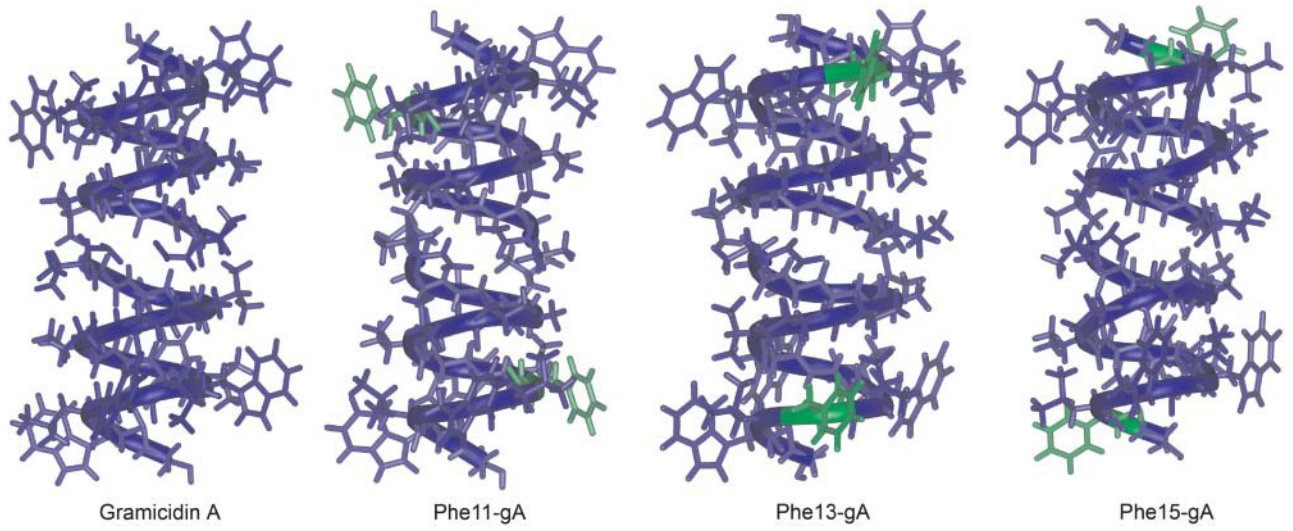

FIGURE 3 Minimized average structures of gA and its (*left* to *right*) Phe11-, Phe13-, and Phe15- analogs. The site of substitution is highlighted in green.

et al., 1993). The kinetic parameters for incorporation of each analog into PC/PG vesicles are shown in Table 3 (Easton, 1989), and RMSD statistics for the 10 lowest energy structures of each analog are given in Table 4. A summary of restraints used in the structure calculation is provided in Table 5, and results of RMSD analyses of the backbones and side chains of each analog compared to native gA are shown in Table 6 and Table 7, respectively.

The effects of Trp→Phe replacements on single-channel conductance

Previous publications regarding the effects of amino acid replacement on the single-channel conductance and the kinetics of ion transport of certain gramicidin analogs have shown that channel function is affected to a greater extent by replacements nearer the NH_2-terminus of the channel (Becker et al., 1991; Hinton et al., 1993, 1997; Hinton and Washburn, 1995; Andersen et al., 1998). Activation enthalpy is a measure of the overall energy barrier to transport of the Na^+ ions through a gramicidin channel from the binding at the channel entrance, translocation through the channel, and to the channel exit (Hinton et al., 1988b). An increase in activation enthalpy is expected to cause a decrease in single-channel conductance. As shown by comparison of Tables 1 and 2, activation enthalpy values for the transport of the Na^+ ion correspond quite well with single-channel conductance studies in that the results of both studies point to a similar trend. The activation enthalpy values determined by NMR illustrate that Na^+ ion transport is more highly affected by replacements located near the binding site (Trp_9 and Trp_{11}) (Easton, 1989). These results complement those obtained by Becker et al. in single-channel conductance studies. The results of the single-channel measurements indicate that the conductance and lifetime of Phe_{13}-gA are very similar to those of Phe_{15}-gA. However, conductance and channel lifetimes for Phe_{11}- and Phe_9-gA differ drastically compared to the other analogs (Table 1); all of these results are complemented by the activation enthalpy studies. The goal of this research is to explain these trends using the three-dimensional structures of the Phe_{11}-, Phe_{13}-, and Phe_{15}-gA channels. A similar observation has been made elsewhere (Hinton and Washburn, 1995; Hinton et al., 1997).

Examination of overlay structures (Fig. 6, *A–C*) and RMSD values (Tables 6 and 7) indicate that only small changes occur in the overall channel structure, even with replacements at position 11 (the most interior of the viable analogs studied). It is obvious that deviations are likely to occur at the replacement sites. Therefore, attention is focused on other side chains, such as those neighboring the substitution sites. The most significant structural variations follow the β-helical model in that differences in side-chain orientation are limited to the residues neighboring the substitution site and those five to seven residues away (Urry, 1984). The backbone folds of each analog (gA, Phe_{11}-gA, Phe_{13}-gA, and Phe_{15}-gA) are very similar, as indicated by the statistical analysis in Table 6. Phe_{15}-gA exhibits side-chain deviations at residues $DLeu_{12}$ and $DLeu_{14}$, and to a lesser extent at $DLeu_4$. Changes in the position of residue 14 appear to be solely due to replacement of the native Trp at position 15. $DLeu_{14}$ is an immediate neighbor to the Trp at position 15 and, as observed in the NOESY spectra, these side chains exert numerous through-space interactions. Removal of Trp at position 15, therefore, is likely to cause a change in the position of the $DLeu_{14}$ side chain through neighboring residue effects (i, $i - 1$ interaction). The distortions observed in the $DLeu_{12}$ and $DLeu_4$ side chains are primarily attributed to inability to resolve the R and S chemical shift assignments of methyl groups on the side chains. These deviations are small ($<$1.5 Å as in Table 6),

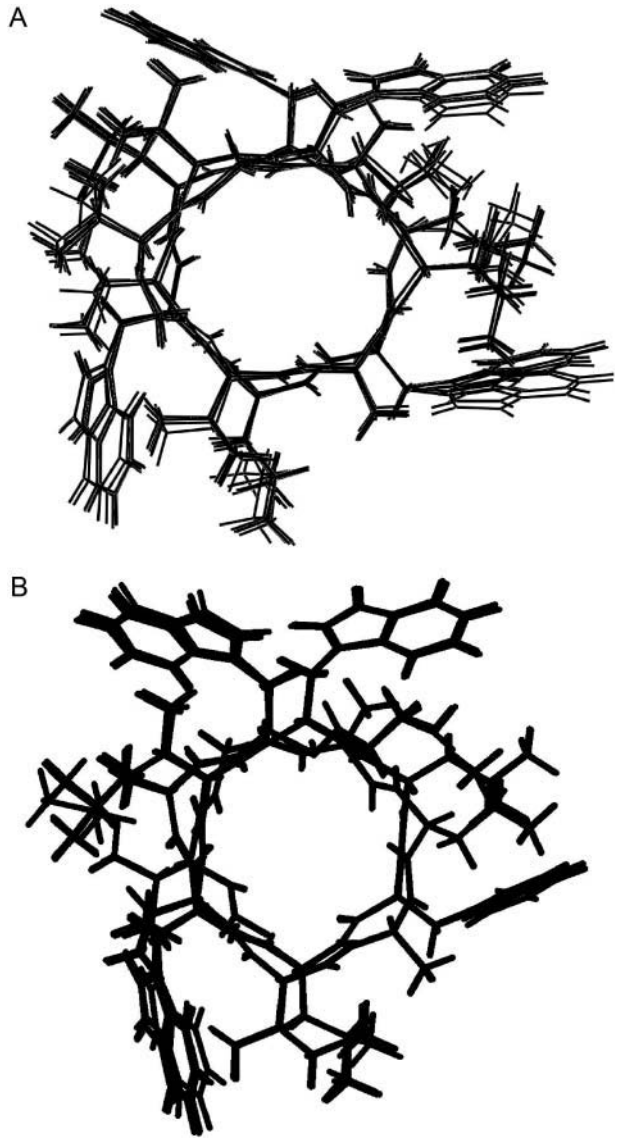

FIGURE 4 Superposition plots of the 10 best conformers of gramicidin A (*A*) and Phe11-gA (*B*) as calculated using Aria 1.2.

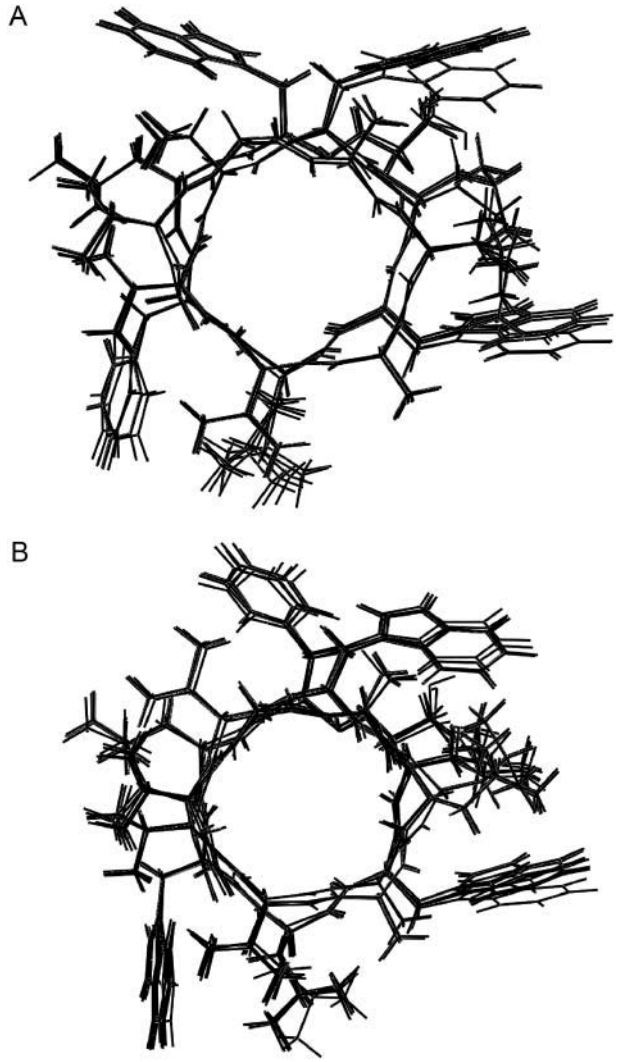

FIGURE 5 Superposition plots of the 10 best conformers of Phe13-gA (*A*) and Phe15-gA (*B*) as calculated using Aria 1.2.

and are likely not real structural effects (Clore et al., 1993; Zhao and Jardetzky, 1994; Spronk et al., 2002).

In Phe_{13}-gA, side-chain deviations are limited to $DVal_8$, $DLeu_{12}$, and $DLeu_{14}$, all of which are expected due to the helical conformation ($i - 5$, $i - 1$, and $i + 1$ interactions). Variations in $DLeu_{12}$ and $DLeu_{14}$ side chains flanking the replacement are likely a direct result of the substitution at position 13. The $DVal_8$ side chain is five residues away from the replacement site, and a small variation observed at this position can be attributed to $i - 5$ interactions through the helix (residues $8 \rightarrow 13$).

The primary differences observed in Phe_{11}-gA are in side-chain positions of $DLeu_{10}$ neighboring the replacement ($i - 1$) site and $DLeu_4$ seven residues away ($i - 7$). These variations are both likely a direct result of the substitution at position 11. In addition, a small deviation is observed in the orientation of the $DLeu_{14}$ side chain. This small variation (<1.5 Å), again, is due to assignment ambiguity in the methylene groups of this particular side chain and is not considered a real structural effect.

From transverse overlay plots, it can be seen that the primary effect on side chains in each analog are present adjacent to the replacement site, or five to seven residues away. In all cases, side-chain RMSD calculations are <2 Å and backbone RMSD values are <1 Å. These values indicate that the backbone fold is highly conserved in each analog and that only small changes in side-chain orientation are observed. From this information, it can be determined that drastic changes in channel structure are not present. Assuming similar behavior of gramicidins in multiple lipid systems (Townsley, 2000), it appears that structural changes alone are not likely to be the cause of reduced channel

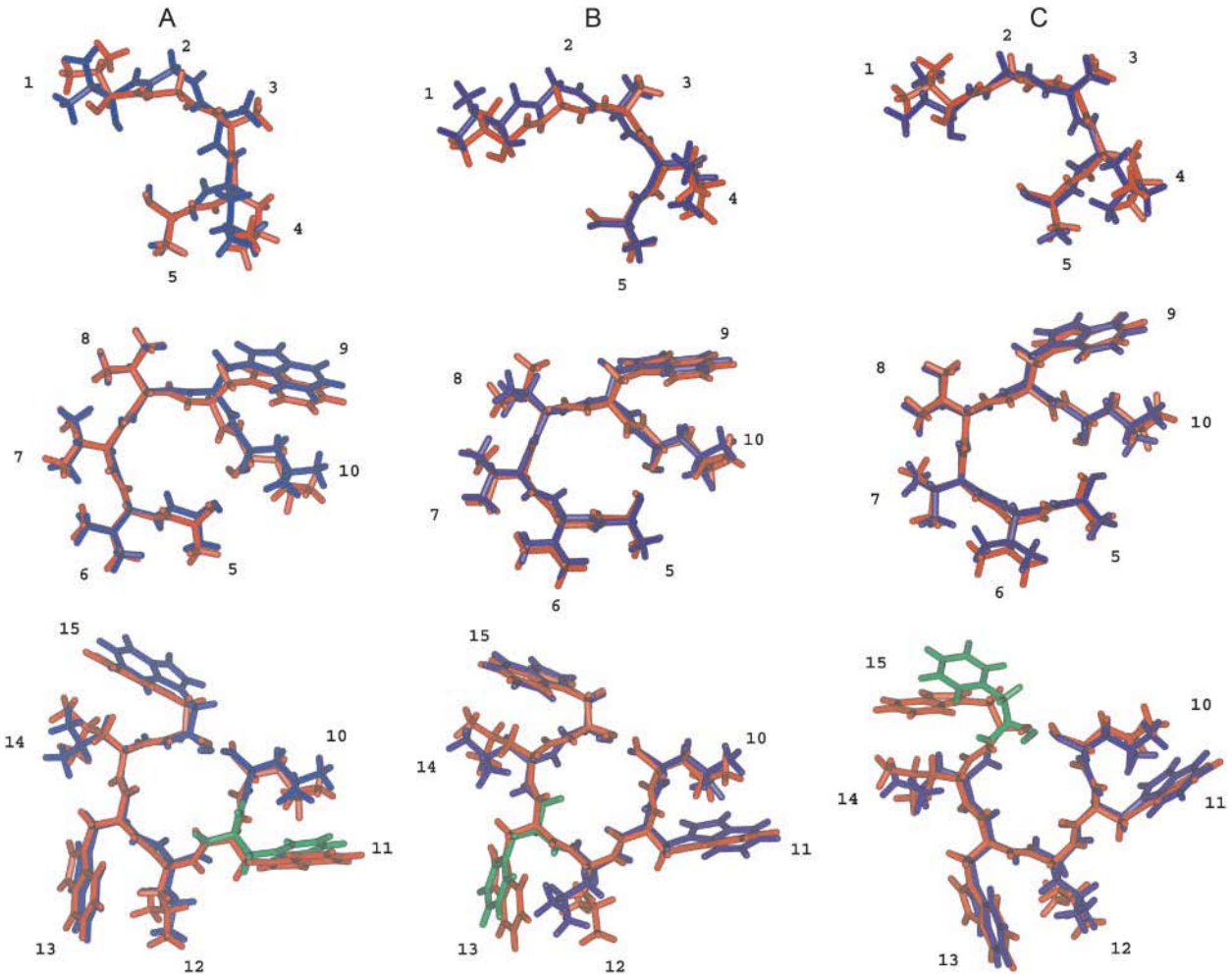

FIGURE 6 Transverse sliced overlay plots of Phe analogs with native gramicidin A. Panels *A*, *B*, and *C* show Phe_{11}-gA, Phe_{13}-gA, and Phe_{15}-gA, respectively, in blue overlaid on native gA in red. The site of replacement in the Phe analogs is highlighted in green.

function as observed in single-channel conductance and transport kinetics studies.

The dipole at residue 15 appears to play a role in guiding the ion into the channel entrance. Removal of this dipole likely alters the ability of the ion to enter the channel, and could result in the decrease in single-channel conductance of this analog. Phe_{13}-gA exhibits a very small increase in single-channel conductance over Phe_{15}-gA, indicating that Trp_{13} has an accompanying role to Trp_{15} in that it assists in transport of the ion into the channel and toward the binding site. Lastly, removal of the Trp dipole by Trp→Phe replacement at positions 11 and 9 results in a drastic decrease (30–40%) in single-channel conductance relative to the other two analogs, an observation supported by the ion transport kinetics studies. Results previously published indicate that, based upon binding and transport NMR data, a direct correlation exists between the strength of ion binding and the transport rate of theyhh specific permeant ion (Hinton et al., 1988b; Hinton, 1999). Conductance and activation enthalpy measurements suggest that the dipoles at positions 9 and 11 have a more significant role in ion binding than do the 13 and 15 residues, a conclusion supported by previously published observations (Hinton et al., 1986; Easton, 1989; Sung and Jordan, 1989; Becker et al., 1991; Hu and Cross, 1995; Tian et al., 1996; Cotten et al., 1999). As previously stated, the Na^+ binding site is believed to lie near the Leu_{10} and Leu_{12} residues (Roux and Karplus, 1991, 1994; Woolf and Roux, 1994). Thus, removal of ion-dipole interactions at position 11 or 9 is likely to inhibit or prevent ion-dipole interactions

TABLE 2 Activation enthalpy (ΔH) and entropy (ΔS) for Na^+ ion transport across PC/PG vesicle membranes by gA and phenylalanine analogs

Gramicidin analog	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (e.u.)
Phe_9-gA	10.6 ± 0.3	7.5 ± 2.1
Phe_{11}-gA	10.1 ± 0.4	6.5 ± 2.2
Phe_{13}-gA	9.6 ± 0.3	5.5 ± 1.3
Phe_{15}-gA	9.2 ± 0.2	5.0 ± 1.5
Gramicidin A	8.5 ± 0.2	4.2 ± 1.2

Data obtained from Easton (1989).

TABLE 3 Activation enthalpy (ΔH) and entropy (ΔS) for incorporation into PC/PG vesicles of gA and phenylalanine analogs

Gramicidin analog	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (e.u.)
Gramicidin A	11.8 ± 0.7	−11 ± 2
Phe_9-gA	13.1 ± 0.4	−8 ± 2
Phe_{11}-gA	14.6 ± 0.4	−4 ± 2
Phe_{13}-gA	15.5 ± 0.5	−1 ± 2
Phe_{15}-gA	17.0 ± 0.7	4 ± 2

Data obtained from Easton (1989).

TABLE 5 Summary of constraints used for the structure calculations of gA and Phe analogs

	Gramicidin A	Phe_{15}-gA	Phe_{13}-gA	Phe_{11}-gA
NOEs				
Intramolecular	576	568	586	548
Intermolecular	22	22	23	23
Per residue	16.9	16.7	17.2	16.1
H-bonds				
Intramolecular	20	20	20	20
Intermolecular	6	6	6	6

necessary for ion binding. These dipole alterations, together with any structural effects of the substitutions, may result in a direct reduction in the ability of the channel to bind and conduct the transported ion. However, Trp→Phe substitutions at the 9, 11, 13, and 15 positions of gA seem to have no effect on the ability of the peptides to form ion-conducting channels (though only the Phe_{11}-, Phe_{13}-, and Phe_{15}- analogs form single, helical species), as evidenced by the data reported in Becker et al. (1991).

Another observation as to orientation of the Phe rings in each analog is reported here. Comparison of Phe-substituted gramicidins with native gA (Fig. 6, *A–C*) shows that even with replacement of the Trp residues, the substituted Phe rings adopt nearly the same conformation as the native Trp rings. This observation suggests that the DLeu ''spacer'' residues (Jude et al., 1999) located at positions 10, 12, and 14 are important in fixing the position of the Trp rings, enabling them to maximize interactions with the membrane bilayer and modulate channel stability.

The effects of Trp→Phe replacements on incorporation and channel lifetime

Residue hydrophobicity is an important factor in the insertion of the gramicidin A channel into lipid bilayers (Becker et al., 1991; Wallace, 2000). Since there is a significant hydrophobicity difference between Trp and Phe (Eisenberg et al., 1984; Engelman et al., 1986; Jacobs and White, 1989; Lesser and Rose, 1990), the Trp→Phe substitutions cause changes in the hydrophobic interactions between the side chains of the residues and the surrounding lipids.

Single-amino acid substitutions can have a significant effect on the incorporation of gramicidin into bilayers, as shown by the differences found in $\Delta H^{\pm}$ and $\Delta S^{\pm}$ of activation for incorporation (Table 3) between the analogs. The data clearly show that gA, with four Trp residues, is more easily incorporated into bilayers (i.e., smallest $\Delta H^{\pm}$) as single-stranded dimers than the Phe-substituted analogs. Furthermore, the results indicate that the position of the substitution also has an effect upon this incorporation; a similar type phenomenon has been observed in previous publications (Becker et al., 1991; Hinton and Washburn, 1995; Hinton et al., 1997).

Side chain-side chain and side chain-lipid interactions play an important role in gramicidin-lipid organization and the ability of the channel to self-associate, and Trp rings are known to be essential for gramicidin's ability to modulate lipid structure (Killian et al., 1985). Fluorescence studies have shown that Trp residues play an important role in the incorporation of gramicidin into lysophosphatidylcholine micelles and the ability of gramicidin to induce the formation of bilayers (Masotti et al., 1980; Spisni et al., 1983). In addition, previous studies have shown that gB exhibits a reduction in the capability of the peptide to modulate lipid structure compared to gA (Killian et al., 1985). Such results demonstrate the importance of side chains in modulating lipid-channel structure and indicate that lipid-side chain interactions could play an important role in the incorporation process. When a sample is raised above the phase-transition

TABLE 4 Structural statistics for 10 lowest energy structures of gramicidin A and Phe analogs

RMSD*	Gramicidin A	Phe_{15}-gA	Phe_{13}-gA	Phe_{11}-gA
Bonds (Å)	0.022	0.022	0.022	0.022
Angles	3.180°	3.180°	3.196°	3.180°
Impropers	8.489	8.677	8.776	8.692

*All RMSD values calculated using the program ARIA 1.2.

TABLE 6 Residue-by-residue backbone RMSD values (Å) of each Phe analog relative to native gramicidin A

Residue	Phe_{15}-gA	Phe_{13}-gA	Phe_{11}-gA
1	0.61	0.97	0.93
2	0.74	0.94	0.80
3	0.57	0.73	0.74
4	0.92	0.32	0.96
5	0.50	0.96	0.53
6	0.42	0.73	0.35
7	0.43	0.37	0.19
8	0.42	0.60	0.32
9	0.55	0.84	0.81
10	0.31	0.72	0.48
11	0.76	0.65	N/A
12	0.90	0.95	0.39
13	0.84	N/A	0.52
14	0.93	0.91	0.34
15	N/A	0.38	0.69

The sites of substitution are omitted due to the presence of different residues at these locations. All RMSD values were calculated using the Crystallography and NMR System of Brunger et al. (1998).

TABLE 7 Residue-by-residue side-chain RMSD values (Å) of each Phe analog relative to native gramicidin A

Residue	Phe_{15}-gA	Phe_{13}-gA	Phe_{11}-gA
1	0.85	0.69	0.94
2	—*	—*	—*
3	0.66	0.58	0.69
4	1.15	0.86	2.04
5	0.48	0.76	0.67
6	0.96	0.51	0.35
7	0.39	0.41	0.37
8	0.73	1.70	0.30
9	0.55	0.48	0.79
10	0.84	0.63	1.66
11	0.93	0.70	N/A
12	1.40	2.37	0.98
13	0.60	N/A	0.64
14	1.72	1.68	1.40
15	N/A	0.47	0.85

The sites of substitution are omitted due to the presence of different residues at these locations. All RMSD values were calculated using the Crystallography and NMR System of Brunger et al. (1998).
*There is no side-chain correlation for these Gly residues.

temperature (≈42°C) so that the membrane (composed of phosphatidylcholine and phosphatidylglycerol) is in a ''liquid'' phase, gramicidin is able to incorporate into the membrane. This phase-transition temperature involves the disruption of intermolecular hydrogen bonds among the polar headgroups of the lipids (Tanford, 1980). To incorporate into the membrane, gramicidin must disrupt the lipid-lipid hydrogen bonds. Upon insertion, the ability of Trp side chains to hydrogen-bond allows gramicidin to compensate for this disruption. Therefore, removal of this ability by Trp → Phe substitutions is likely to cause a significant effect on the ability of the analog to incorporate and stabilize the bilayer. Previous work involving N-methylation of Trp residues in gramicidin (Sun et al., 2002) has indicated that removal of the hydrogen-bonding ability of Trp residues alters the distribution of gramicidin conformers, resulting in a more highly favored double-stranded conformation. However, singly substituted Phe- analogs of gA do not exhibit this preference, as indicated by the presence of a predominantly single-stranded helical species in solutions used for structure determination.

It should be noted that a fundamental difference exists between the kinetics of incorporation and the measurement of average channel lifetimes. Although determination of incorporation kinetics gives insight into the ability of the peptide to insert into the bilayer, the measurement of average channel lifetimes actually provides a measure of the ability of two monomer species to associate/dissociate after they have incorporated into the bilayer. Results presented here indicate that there are no significant structural differences among the three analogs that would contribute to observed decreases in channel lifetimes. These results, therefore, support those of Becker et al. (1991) in that the variation in hydrophobic character of position 11 has a direct effect on the single-channel lifetime. This is evidenced by results of the average channel lifetime experiments shown in Table 1.

The channel lifetime results presented in Becker et al. appear to represent a very complex relationship between the channel and the bilayer. Trp residues are amphipathic, and have portions that can interact with polar and nonpolar environments. One would expect that if a Trp residue existed near the channel entrance (at the membrane/aqueous interface—where interaction with bulk water seems more likely), it is possible that the hydrophilic region (the indole-NH) will be able to hydrogen-bond to external H_2O, whereas its nonpolar surface remains buried in the membrane. Previously published data indicate that Trp_9 and Trp_{11} of native gA do not supply any sites available for solvent exchange, suggesting that these two Trp residues exist in the more hydrophobic portion of the micelle (Hinton, 1996). It is likely, then, that Trp residues more near the aqueous/membrane interface (Trp_{15} and Trp_{13}) that interact with bulk water would enhance channel lifetime by possessing some hydrophilic properties, a conclusion supported by the incorporation data from Easton (1989). Previous publications involving molecular dynamics simulations of gramicidin in a lipid environment have demonstrated that Trp_{13} and Trp_{15} are more likely to interact with the polar headgroups of the bilayer, whereas Trp_{11} and Trp_9 tend to lie in the more hydrophobic hydrocarbon region (Chiu et al., 1999; Roux and Karplus, 1994). It seems, then, that the presence of a Trp at position 11 might actually result in an energetically unfavorable environment around the hydrophilic portion of the indole ring due to the cost of burying the indole-NH in the nonpolar region of the bilayer. Replacement of Trp with Phe reduces this instability by removing the indole-NH and introducing more favorable hydrophobic-hydrophobic interactions. This could explain why a Trp → Phe substitution at position 11 was found to always increase the channel lifetime in singly and multiply substituted Phe analogs of gA (Becker et al., 1991). This conclusion is also supported by the studies using Trp N-methylation in which the channel lifetimes of the 1-Me-Trp_{11} were longer than those observed for native gA (Sun, 2002). The results obtained with Phe_9, however, appear to be more complex, and invariably represent a more complex problem; one depending on multiple side chain-lipid and side chain-side chain interactions (i.e., Trp_9–Trp_{15}), a hypothesis suggested by Becker et al. (1991).

CONCLUSION

Four analogs of gramicidin A have been synthesized in which the Trp residues at positions 9, 11, 13, and 15 were sequentially replaced by phenylalanine. Three analogs (Phe_{11}-, Phe_{13}-, and Phe_{15}-gA) incorporated into SDS micelles of varying concentrations and formed single helical species whose three-dimensional structures were determined. The replacement of Trp residues did not significantly alter the backbone structure of the channel relative to the

native species. However, previously determined data indicated that removal of the Trp residues induced a significant effect on ion transport properties (Hinton et al., 1993; Becker et al, 1991), channel lifetime (Becker et al., 1991), and the ability of the peptides to incorporate into the bilayer (Easton, 1989). Functional effects were more pronounced with replacement of Trp_{11} and Trp_9, which are believed to be more important in the binding of monovalent ions. Therefore, it is concluded that the absence of the long-range ion-dipole interactions at the channel entrance (positions 15 and 13) causes a decrease in the ability of ion to enter into the channel, whereas removal of the dipoles at positions 11 and 9 is likely to cause a decrease in the ion-binding ability of the channel; resulting in a larger effect on single-channel conductance and transport kinetics. In addition, introduction of a more hydrophobic environment at position 11 (through Trp→Phe substitution) increases the favorability of side chain-lipid interactions in the hydrophobic region of the bilayer. This results in an increase in the channel lifetime of the Phe_{11}- analog relative to Phe_{13}-gA, Phe_{15}-gA, and native gA species. Additional hydrophobicity effects are observed with residues closer to the C-terminus, and appear to affect the ability of the peptide to incorporate into the bilayer.

Funding for this project was provided by National Science Foundation grant MCB-9313835 and National Institutes of Health grant 5P20RR15569-01.

REFERENCES

Andersen, O. S., D. V. Greathouse, L. L. Providence, M. D. Becker, and R. E. Koeppe, Ii. 1998. Importance of tryptophan dipoles for protein function: 5-fluorination of tryptophans in gramicidin A channels. *J. Am. Chem. Soc.* 120:5142–5146.

Anderson, D. G., R. B. Shirts, T. A. Cross, and D. D. Busath. 2001. Noncontact dipole effects on channel permeation. V. Computed potentials of fluorinated gramicidin. *Biophys. J.* 81:1255–1264.

Arseniev, A. S., I. L. Barsukov, V. F. Bystrov, A. L. Lomize, and A. Ovchinnikov Yu. 1985. Proton NMR study of gramicidin A transmembrane ion channel. Head-to-head right-handed, single-stranded helices. *FEBS Lett.* 186:168–174.

Becker, M. D., D. V. Greathouse, R. E. Koeppe II, and O. S. Andersen. 1991. Amino acid sequence modulation of gramicidin channel function: effects of tryptophan-to-phenylalanine substitutions on the single-channel conductance and duration. *Biochemistry.* 30:8830–8839.

Braunschweiler, L., and R. R. Ernst. 1983. Coherence transfer by isotropic mixing: Application to proton correlation spectroscopy. *J. Magn. Reson.* 53:521–528.

Brunger, A. T., P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kuntsleve, J. S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson, and G. L. Warren. 1998. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr.* D54:905–921.

Busath, D. D., C. D. Thulin, R. W. Hendershot, L. R. Phillips, P. Maughan, C. D. Cole, N. C. Bingham, S. Morrison, L. C. Baird, R. J. Hendershot, M. Cotten, and T. A. Cross. 1998. Noncontact dipole effects on channel permeation. I. Experiments with (5-F-indole) Trp13 gramicidin A channels. *Biophys. J.* 75:2830–2844.

Buster, D. C., J. F. Hinton, F. S. Millett, and D. S. Shungu. 1988. 23Na NMR investigation of gramicidin-induced ion transport through membranes under equilibrium conditions. *Biophys. J.* 53:145–152.

Chiu, S., S. Subramaniam, and E. Jakobsson. 1999. Simulation study of a gramicidin/lipid bilayer system in excess water and lipid I. Structure of the molecular complex. *Biophys. J.* 76:1929–1938.

Clore, G. M., M. A. Robien, and A. M. Gronenborn. 1993. Exploring the limits of precision and accuracy of protein structures determined by nuclear magnetic resonance spectroscopy. *J. Mol. Biol.* 231:82–102.

Cole, C. D., A. S. Frost, N. Thompson, M. Cotten, T. A. Cross, and D. D. Busath. 2002. Noncontact dipole effects on channel permeation. VI. 5-F- and 6-F-Trp gramicidin channel currents. *Biophys. J.* 83:1974–1986.

Cotten, M., C. Tian, D. D. Busath, R. B. Shirts, and T. A. Cross. 1999. Modulating dipoles for structure-function correlations in the gramicidin A channel. *Biochemistry.* 38:9185–9197.

Dorigo, A. E., D. G. Anderson, and D. D. Busath. 1999. Noncontact dipole effects on channel permeation. II. Trp conformations and potentials in gramicidin A. *Biophys. J.* 76:1897–1908.

Durkin, J. T., R. E. Koeppe II, and O. S. Andersen. 1990. Energetics of gramicidin hybrid channel formation as a test for structural equivalence. Side-chain substitutions in the native sequence. *J. Mol. Biol.* 211:221–234.

Easton, P. L. 1989. Phenylalanine analogs of gramicidin: incorporation into and cation transport across lipid vesicle membranes. PhD thesis, University of Arkansas, Fayetteville, Arkansas.

Easton, P. L., J. F. Hinton, and D. K. Newkirk. 1990. Kinetics of channel formation of gramicidins A and B in phospholipid vesicle membranes. *Biophys. J.* 57:63–69.

Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* 179:125–142.

Engelman, D. M., T. A. Steitz, and A. Goldman. 1986. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* 15:321–353.

Etchebest, C., and A. Pullman. 1985. The effect of the amino-acid side chains on the energy profiles for ion transport in the gramicidin A channel. *J. Biomol. Struct. Dyn.* 2:859–870.

Henderson, R., J. M. Baldwin, T. A. Ceska, R. Zemlin, E. Beckmann, and K. H. Downing. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* 213: 899–929.

Hinton, J. F. 1996. Cation-binding location and hydrogen exchange sites for gramicidin in SDS micelles using NOESY NMR. *J. Magn. Reson. B.* 112:26–31.

Hinton, J. F. 1999. NMR studies of ion-transporting biological channels. *Annu. Rep. NMR Spectrosc.* 38:89–120.

Hinton, J. F., D. C. Buster, P. Easton, and T. Privett. 1988a. Metal ion NMR study of cation transport through the gramicidin channel. *Prog. Clin. Biol. Res.* 273:331–336.

Hinton, J. F., D. C. Buster, J. Q. Fernandez, T. A. Privett, P. L. Easton, and D. K. Newkirk. 1988b. Thermodynamics of cation binding and transport by gramidicin. *Proc. Jerusalem Symp. Quantum Chem. Biochem. Transp. Membr.* 21:203–218.

Hinton, J. F., P. L. Easton, D. K. Newkirk, and D. C. Shungu. 1993. ^{23}Na-NMR study of ion transport across vesicle membranes facilitated by phenylalanine analogs of gramicidin. *Biochim. Biophys. Acta.* 1146:191–196.

Hinton, J. F., J. B. Jordan, and E. Horne. 2002. Molecular structure heterogeneity of gramicidin analogs incorporated into SDS micelles: a NMR study. *J. Mol. Struct.* 602–603:245–256.

Hinton, J. F., and A. M. Washburn. 1995. Species heterogeneity of Gly-11 gramicidin A incorporated into sodium dodecyl sulfate micelles. *Biophys. J.* 69:435–438.

Hinton, J. F., A. M. Washburn-McCain, A. Snow, and J. Douglas. 1997. Effects of alanine and glycine substitution for tryptophan on the heterogeneity of gramicidin A analogs in micelles. *J. Magn. Reson.* 124:132–139.

Hinton, J. F., W. L. Whaley, D. Shungu, R. E. Koeppe II, and F. S. Millett. 1986. Equilibrium binding constants for the group I metal cations with

gramicidin-A determined by competition studies and thallium-205(1+) nuclear magnetic resonance spectroscopy. *Biophys. J.* 50:539–544.

Hu, W., and T. A. Cross. 1995. Tryptophan hydrogen bonding and electric dipole moments: Functional roles in the gramicidin channel and implications for membrane proteins. *Biochemistry.* 34:14147–14155.

Hu, W., N. D. Lazo, and T. A. Cross. 1995. Tryptophan dynamics and structural refinement in a lipid bilayer environment: Solid state NMR of the gramicidin channel. *Biochemistry.* 34:14138–14146.

Hu, W., K. C. Lee, and T. A. Cross. 1993. Tryptophans in membrane proteins: indole ring orientations and functional implications in the gramicidin channel. *Biochemistry.* 32:7035–7047.

Jacobs, R. E., and S. H. White. 1989. The nature of the hydrophobic binding of small peptides at the bilayer interface: implications for the insertion of transbilayer helices. *Biochemistry.* 28:3421–3437.

Jeener, J., B. H. Meier, P. Bachmann, and R. R. Ernst. 1979. Investigation of exchange processes by 2D NMR spectroscopy. *J. Chem. Phys.* 71: 4546–4563.

Jude, A. R., D. V. Greathouse, R. E. Koeppe II, L. L. Providence, and O. S. Andersen. 1999. Modulation of gramicidin channel structure and function by the aliphatic ''spacer'' residues 10, 12 and 14 between the tryptophans. *Biochemistry.* 38:1030–1039.

Killian, J. A., J. W. Timmermans, S. Keur, and B. De Kruijff. 1985. The tryptophans of gramicidin are essential for the lipid structure modulating effect of the peptide. *Biochim. Biophys. Acta.* 820:154–156.

Koeppe II, R. E., J. A. Killian, and D. V. Greathouse. 1994. Orientations of the tryptophan 9 and 11 side chains of the gramicidin channel based on deuterium NMR spectroscopy. *Biophys. J.* 66:14–24.

Koeppe II, R. E., J.-L. Mazet, and O. S. Andersen. 1990. Distinction between dipolar and inductive effects in modulating the conductance of gramicidin channels. *Biochemistry.* 29:512–520.

Koeppe II, R. E., J. A. Killian, D. V. Greathouse, and O. S. Andersen. 1998. Tryptophan anchors in transmembrane peptides. *Biol. Skr. Dan. Vid. Selsk.* 49:93–98.

Lesser, G. J., and G. D. Rose. 1990. Hydrophobicity of amino acid subgroups in proteins. *Proteins.* 8:6–13.

Linge, J. P., M. Habeck, W. Rieping, and M. Nilges. 2003. ARIA: automated NOE assignment and NMR structure calculation. *Bioinformatics.* 19:315–316.

Masotti, L., A. Spisni, and D. W. Urry. 1980. Conformational studies on the gramicidin A transmembrane channel in lipid micelles and liposomes. *Cell Biophys.* 2:241–254.

Mazet, J. L., O. S. Andersen, and R. E. Koeppe II. 1984. Single-channel studies on linear gramicidins with altered amino acid sequences. A comparison of phenylalanine, tryptophan, and tyrosine substitutions at positions 1 and 11. *Biophys. J.* 45:263–276.

Phillips, L. R., C. D. Cole, R. J. Hendershot, M. Cotten, T. A. Cross, and D. D. Busath. 1999. Noncontact dipole effects on channel permeation. III. Anomalous proton conduction effects in gramicidin. *Biophys. J.* 77:2492–2501.

Piantini, U., O. W. Sorensen, and R. R. Ernst. 1982. Multiple quantum filters for elucidating NMR coupling networks. *J. Am. Chem. Soc.* 104:6800–6801.

Roux, B., and M. Karplus. 1991. Ion transport in a model gramicidin channel. Structure and thermodynamics. *Biophys. J.* 59:961–981.

Roux, B., and M. Karplus. 1994. Molecular dynamics simulations of the gramicidin channel. *Annu. Rev. Biophys. Biomol. Struct.* 23:731–761.

Russell, E. W. B., L. B. Weiss, F. I. Navetta, R. E. Koeppe II, and O. S. Andersen. 1986. Single-channel studies on linear gramicidins with altered amino acid side chains. Effects of altering the polarity of the side chain at position 1 in gramicidin A. *Biophys. J.* 49:673–686.

Sawyer, D. B., L. P. Williams, W. L. Whaley, R. E. Koeppe II, and O. S. Andersen. 1990. Gramicidins A, B, and C form structurally equivalent ion channels. *Biophys. J.* 58:1207–1212.

Schiffer, M., C. H. Chang, and F. J. Stevens. 1992. The functions of tryptophan residues in membrane proteins. *Protein Eng.* 5:213–214.

Sham, S. S., J. Q. Fernandez, L. E. Townsley, D. V. Greathouse, O. S. Andersen, and J. F. Hinton. 2003. The structure, cation binding and transport of Gly15-gramicidin A. *Biochemistry.* 42:1401–1409.

Spisni, A., I. Pasquali-Ronchetti, E. Casali, L. Lindner, P. Cavatorta, L. Masotti, and D. W. Urry. 1983. Supramolecular organization of lysophosphatidylcholine-packaged gramicidin A″. *Biochim. Biophys. Acta.* 732:58–68.

Spronk, C. A., J. P. Linge, C. W. Hilbers, and G. W. Vuister. 2002. Improving the quality of protein structures derived by NMR spectroscopy. *J. Biomol. NMR.* 22:281–289.

Sun, H. 2002. Applications of gramicidin channels: I. Function of tryptophan at the membrane/water interface. II. Molecular design of membrane-spanning force transducers. PhD thesis, University of Arkansas, Fayetteville, Arkansas.

Sun, H., E. M. Scherer, S. Shobana, D. V. Greathouse, R. E. Koeppe II, and O. S. Andersen.2002. Properties of gramicidin A channels with modified tryptophan indole rings. *Biophys. J.* 82:556A.

Sung, S. S., and P. C. Jordan. 1989. The channel properties of possible gramicidin dimers. *J. Theor. Biol.* 140:369–380.

Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes, John Wiley & Sons, New York.

Thompson, N., G. Thompson, C. D. Cole, M. Cotten, T. A. Cross, and D. D. Busath. 2001. Noncontact dipole effects on channel permeation. IV. Kinetic model of 5-F-Trp13 gramicidin A currents. *Biophys. J.* 81:1245–1254.

Tian, F., K. C. Lee, W. Hu, and T. A. Cross. 1996. Monovalent cation transport: lack of structural deformation upon cation binding. *Biochemistry.* 35:11959–11966.

Townsley, L. 2000. The three-deminsional structure of gramicidin analogs in micellar environments determined using two-dimensional nuclear magnetic resonance spectroscopic techniques. PhD thesis, University of Arkansas, Fayetteville, Arkansas, USA.

Townsley, L. E., W. A. Tucker, S. Sham, and J. F. Hinton. 2001. Structures of gramicidins A, B, and C incorporated into sodium dodecyl sulfate micelles. *Biochemistry.* 40:11676–11686.

Tredgold, R. H., and P. N. Hole. 1976. Dielectric behaviour of dry synthetic polypeptides. *Biochim. Biophys. Acta.* 443:137–142.

Urry, D. W. 1984. Molecular structure of the gramicidin transmembrane channel: utilization of carbon-13 nuclear magnetic resonance, ultraviolet absorption, circular dichroism and infrared spectroscopies. *In* Spectroscopy of Biological Molecules. C. Sandorfy and T. Theophanides, editors. D. Reidel Publishing, Dordrecht, The Netherlands. 487–510.

Urry, D. W., M. C. Goodall, J. D. Glickson, and D. F. Mayers. 1971. The gramicidin A transmembrane channel: characteristics of head-to-head dimerized π(L,D) helices. *Proc. Natl. Acad. Sci. USA.* 68:1907–1911.

Wallace, B. A. 2000. Common structural features in gramicidin and other channels. *Bioessays.* 22:227–234.

Wallace, B. A., and R. W. Janes. 1999. Tryptophans in membrane proteins: X-ray crystallographic analyses. *Adv. Exp. Med. Biol.* 467:789–799.

Williams, L. P., E. J. Narcessian, O. S. Andersen, G. R. Waller, M. J. Taylor, J. P. Lazenby, J. F. Hinton, and R. E. Koeppe II. 1992. Molecular and channel-forming characteristics of gramicidin K″s: A family of naturally occurring acylated gramicidins. *Biochemistry.* 31:7311–7319.

Woolf, T. B., and B. Roux. 1994. Molecular dynamics simulation of the gramicidin channel in a phospholipid bilayer. *Proc. Natl. Acad. Sci. USA.* 91:11631–11635.

Yau, W.-M., W. C. Wimley, K. Gawrisch, and S. H. White. 1998. The preference of tryptophan for membrane interfaces. *Biochemistry.* 37: 14713–14718.

Zhao, D., and O. Jardetzky. 1994. An assessment of the precision and accuracy of protein structures determined by NMR: Dependence on distance errors. *J. Mol. Biol.* 239:601–607.