Conformation States of Gramicidin A along the Pathway to the Formation of Channels in Model Membranes Determined by 2D NMR and Circular Dichroism Spectroscopy†

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ABSTRACT: Gramicidin A incorporated into SDS (sodium dodecyl sulfate) micelles exists as a right-handed, N-to-N-terminal $\beta^{6.3}$ helical dimer [Lomize, A. L., Orechov, V. Yu., & Arseniev, A. S. (1992) *Bioorg. Khim. 18*, 182–189]. In the incorporation procedure to achieve the ion channel state of gramicidin A in SDS micelles, trifluoroethanol (TFE) is used to solubilize the hydrophobic peptide before addition to the aqueous/micelle solution. The conformational transition of gramicidin A to form ion channels in SDS micelles, i.e., in TFE and 10% TFE/water, has been investigated using 2D NMR and CD spectroscopy. In neat TFE, gramicidin A was found to be monomeric and may possibly exist in an equilibrium of rapidly interconverting conformers of at least three different forms believed to be left- and/or right-handed α and $\beta^{4.4}$ helices. It was found that the interconversion between these conformers was slowed down in 55% TFE as evident by the observation of at least three different sets of $d_{\alpha N}$ COSY peaks although CD gave a net spectrum similar to that in neat TFE. In 10% TFE gramicidin A spontaneously forms a precipitate. The precipitated species were isolated and solubilized in dioxane where gramicidin conformers undergo very slow interconversion and could be characterized by NMR. At least seven different gramicidin A conformations were found in 10% TFE. Four of these are the same types of double helices as previously found in ethanol (i.e., a symmetric left-handed parallel $\beta^{5.6}$ double helix, an unsymmetric left-handed parallel $\beta^{5.6}$ double helix, a symmetric left-handed antiparallel $\beta^{5.6}$ double helix, a symmetric right-handed parallel $\beta^{5.6}$ double helix); the fifth is possibly a symmetric right-handed antiparallel $\beta^{5.6}$ double helix. There is also evidence for the presence of at least one form of monomeric species. Previous observation on the solvent history dependence in the ease of channel incorporation may be explained by the presence of several different folding pathways to channel formation. To test this proposal, the conformation of gramicidin A in 10% DMSO and 10% methanol was studied. In the former environment, the major form was a random coil with a minor population of double-stranded helices, while in the latter, NMR spectra indicate the presence of the same double-helical conformers as found in neat methanol.

Gramicidin A is a 15-residue hydrophobic peptide that forms transmembrane channels which are selective for the transport of monovalent cations (Hladky & Haydon, 1970, 1972; Myers & Haydon, 1972). It has the sequence HCO-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-D-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-$NHCH_2CH_2OH$ (Sarges & Witkop, 1965). Although many problems concerning the structure and properties of the gramicidin A channel have been already addressed extensively, very few studies have been directed toward understanding the process leading to gramicidin channel formation. Therefore, the objective of the present study was to investigate the gramicidin folding process and to determine the intermediates involved during the incorporation of gramicidin A into the channel state in a model membrane environment.

Gramicidin A is known to adopt various conformations depending on its environment. In the single-stranded $\beta^{6.3}$ helical dimer channel structure (Urry, 1971) two gramicidin molecules are jointed at their NH_2 termini and rolled into a β-helix that spans the lipid bilayer. Although there is no crystal structure for this channel conformation, studies using solid-state NMR and other spectroscopic methods have shown that this $\beta^{6.3}$ helical dimer is the predominant thermodynamically stable structure in lipid membranes and SDS (sodium dodecyl sulfate) micelles and is right-handed (Cornell et al., 1988; Smith et al., 1989; Davis, 1988; Nicholson et al., 1987; Bystrov et al., 1987; Lomize et al., 1992; Nicholson & Cross, 1989; Koeppe et al., 1992; Ketchem et al., 1993). The presence of a minor population (5%–10%) of membrane-incorporated monomers of left- and right-handedness has also been proposed in several studies (Sawyer et al., 1989, 1990; Koeppe et al., 1992). In organic solutions and crystals grown from organic solvents gramicidin A adopts various forms of intertwined, double-stranded structures. Two-dimensional NMR (2D NMR) studies by Bystrov and Arseniev (1988) revealed a mixture of four different conformations of intertwined $\beta^{5.6}$ double-stranded helices that interconvert in ethanol. These conformers, which differ from each other in handedness and the "stagger" of their polypeptide chains, have been predicted previously by Veatch et al. (1974) on the basis of infrared (IR), circular dichroism (CD), and thin-layer chromatography (TLC). X-ray diffraction studies have obtained two types of double-stranded β-helix structures of gramicidin A, the antiparallel $\beta^{5.6}$ helix grown in benzene/ethanol azeotrope (Langs, 1988) and the antiparallel $\beta^{6.4}$ helix (Wallace & Ravikumar, 1988) gramicidin–CsCl complex grown in methanol solution. Another form of double-stranded structure, the right-handed $\beta^{7.2}$ helix, was obtained for the gramicidin–cesium

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thiocyanate complex in a 50:50 methanol/chloroform mixture using 2D NMR (Arseniev et al., 1985). Other possible stable structures such as the α and $\beta^{4,4}$ helices have been predicted by conformational energy calculations and studies utilizing a variety of physicochemical techniques on synthetic peptides with alternating L and D stereochemistry (Colonna-Cesari et al., 1977; Hesselink & Scheraga, 1972; Ramachandran & Chandrasekaran, 1972).

A solvent history dependence has been suggested for the ultimate formation of the channel state in membranes (Killian et al., 1988; LoGrasso et al., 1988). While incorporation of gramicidin into the $\beta^{6.3}$ channel form in preformed micelles or lipid membranes from a solution of trifluoroethanol (TFE) or dimethyl sulfoxide (DMSO) can be achieved directly (that is, without sonication and/or heating) (Killian et al., 1988), addition of gramicidin A from a solution of ethanol, chloroform/methanol, and benzene/methanol requires sonication accompanied by extensive incubation (Wallace, 1990). In order to explain this ease of incorporating gramicidin into model membranes as the channel form using TFE and DMSO, it has been proposed that gramicidin adopts a $\beta^{6.3}$ helical monomer in both of these solvents. However, an NMR study by Roux et al. (1990) has demonstrated that gramicidin is a random coil in DMSO. However, before the present work the conformation of gramicidin A in TFE was still questionable.

Single-channel conductance studies by O'Connell et al. (1990) indicated that the majority of gramicidin A channels were formed via insertion of $\beta^{6.3}$ helical monomers, which initially adsorbed onto each side of the lipid monolayer and then dimerized to form the channel structure. Interestingly, the study also provided evidence for channel formation that proceeded through the insertion of intertwined double helices which have to unwind to form the $\beta^{6.3}$ helical dimer channels. In a recent study, Zhang et al. (1992) suggested that gramicidin A introduced from a benzene/ethanol solvent system formd channels through the rearrangement of a left-handed intertwined double helix starting structure.

When channel incorporation is achieved by addition of gramicidin A from organic solvents into preformed lipid membranes or SDS micelles, gramicidin A undergoes a conformational transition from a solubilized form in organic medium to precipitated species in aqueous medium and ultimately, upon sonication, into the channel form in micelles or the lipid bilayer. The formation of precipitate is exacerbated at high gramicidin concentration. Hence, at concentrations of ~3–5 mM used for NMR conformational studies, incorporation into SDS micelles can only be achieved using TFE. In our approach to understand the folding process that led to channel formation in SDS micelles, we have characterized the conformational state of gramicidin A in TFE and 10% TFE using 2D NMR and CD. Since gramicidin A forms a precipitate in 10% aqueous TFE, the conformation of gramicidin A in this solution was studied by isolating the precipitate and dissolving it in dioxane. In dioxane, gramicidin has been shown to retain its conformation(s) for at least 2 weeks at room temperature and even longer at a lower temperature before it undergoes interconversion (Veatch et al., 1974; Pascal et al., 1992; the present study). In addition, the effect of solvent history on the folding process into the channel state has also been investigated using DMSO and methanol.

To data there has not been much, if any, published data on the conformational state of gramicidin A in TFE or in an aqueous solution. Hence, results from the present studies will provide the characterization of the gramicidin A conformation in TFE and in the transitional organic–aqueous environment prior to the formation of channels. In light of the present findings, together with the experimental observations made in other studies, a model has been proposed to describe the mechanistic process of gramicidin folding into the channel form in model membranes. In addition, factors that result in solvent dependence on the ease of channel formation will be discussed.

MATERIALS AND METHODS

Sample Preparation. Gramicidin A was separated and purified from gramicidin D (Sigma Chemical Co., St. Louis, MO) by a procedure described previously (Koeppe & Weiss, 1981). All of the solvents used in the NMR experiments were purchased from Cambridge Isotopes Laboratories (Cambridge, MA). Gramicidin A in TFE was prepared by dissolving purified and dried gramicidin A in 0.7 mL of TFE-d_2 (CF_3-CD_2OH) to a concentration of 50 mM. In the preparation of the gramicidin A precipitate from 10% TFE for NMR studies, the conditions used for channel incorporation into SDS micelles were followed. The peptide was first solubilized in 0.1 mL of TFE-d_2 to a concentration of 50 mM, followed by addition to 0.9 mL of deionized water where a precipitate was formed. This precipitate was sedimented by centrifugation followed by removal of the supernatant. Without further drying the precipitate was dissolved in dioxane-d_8 (to "freeze" the conformations) to a final volume of 0.6 mL. The sample was stored at −20 °C until ready for use. Gramicidin A precipitated from 10% DMSO and 10% methanol dissolved in dioxane was prepared in a similar manner.

Circular Dichroism Measurements. CD spectra were obtained on a Jasco J-700 series spectropolarimeter (Jasco Inc., Easton, MD). All measurements were made at 25 °C in a quartz cuvette of 0.1-cm path length with sample concentrations between 30 and 100 mM. Sample concentration was determined by weighing dry gramicidin A powder and carrying out appropriate dilutions. Spectra were recorded over the wavelength range of 190–280 nm with a 1-s time constant, spectral steps of 0.2 nm, and a scan rate of 50 nm/min. Between six and eight scans were averaged. CD measurements were expressed as mean residue ellipticities, $[\Theta]$, in deg cm^2 dmol^{-1}. Conversion from observed ellipticities, (θ), in millidegrees, was performed by using the relationship $[\Theta] = (\theta)/10cnl$, where c is the peptide concentration in molar, n is the number of amino acid residues, and l is the path length of the cell in centimeters. Secondary structures were determined by direct comparison with CD spectra for gramicidin A in organic solvents and membrane environments (Veatch et al., 1974; Wallace, 1986; Wallace et al., 1981). It should be noted that the net CD spectrum of the gramicidin A equilibrium mixture of interconverting conformers or the spectra of the individual gramicidin A conformers do not correspond to those obtained for typical α-helical or β-sheet secondary structures. This is due to two features of the gramicidin primary structure; the first is the alternating L- and D-amino acids, which results in different ϕ, ψ angles for the polypeptide backbone, and the second is that the high tryptophan content (4 out of 15 amino acids) tends to obscure the peptide's transitions and dominate the gramicidin far-UV CD spectra in the region around 222 nm (Wallace, 1986).

NMR Measurements. ^{1}H NMR spectra were obtained with a Varian VXR-500S NMR spectrometer. Data were collected at temperatures ranging from 10 to 30 °C. All samples were filtered prior to being transferred into a 5-mm NMR sample tube (Wilmad Glass Co., 535-PP grade). The observed ^{1}H chemical shifts were referenced to internal tetramethylsilane (TMS). Spectral widths ranged from 5000

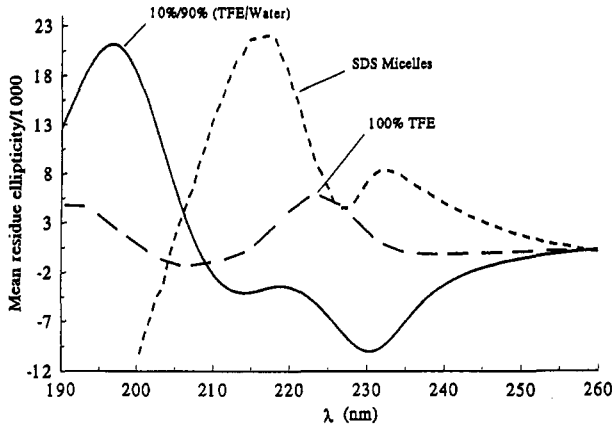


FIGURE 1: CD spectra of gramicidin A in neat TFE, 10% TFE/water solution, and SDS micelles at 25 °C. The peptide concentration was 100 μM.

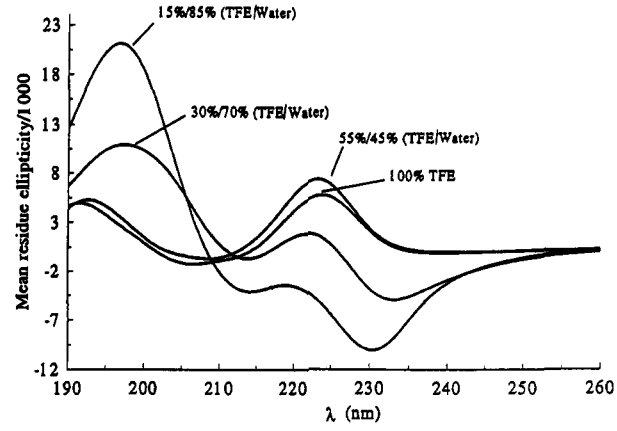


FIGURE 2: CD spectra of gramicidin A in varying ratios of TFE/water at 25 °C. The spectra show a change characteristic of intertwined double-helical structure (positive ellipticity around 197 nm and negative ellipticity around 230 nm) as the concentration of TFE is decreased. The peptide concentration was 100 μM.

to 8000 Hz and were set equally in both dimensions in the 2D experiments. Water suppression was achieved by transmitter presaturation. All spectra were acquired in the phase-sensitive mode (Bodenhausen et al., 1984) using the States, Ruben, and Haberkorn method (States et al., 1982). Except for DQF-COSY, 512 t_1 increments (256 imaginary and 256 real data points) and 4096 t_2 data points were collected in all experiments. DQF-COSY spectra were acquired in 512 t_1 increments with 8192 or 16 384 t_2 data points to achieve a high digital resolution in the F_2 dimension for measurement of $^3J_{HNC\alpha}$ couplings. Between 16 and 128 transients were used per t_1 increment. HOHAHA experiments were collected at mixing times of 50, 65, 75, and 80 ms [with the MLEV-17 mixing scheme (Bax & Davis, 1985)]. NOESY experiments were collected at mixing times of 80 and 200 ms while the mixing time used in ROESY was 200 ms. NMR data sets were processed on a Sun 4/110 Sparcstation using the VNMR software program (Varian Associates). Shifted sine-bell and squared sine-bell window functions were used in both dimensions in the processing of the 2D data sets. With the exception of DQF-COSY, all data sets were zero-filled to 8K and 2K in the F_2 and F_1 dimensions, respectively. DQF-COSY data sets collected with high digital resolution in the F_2 dimension were zero-filled to 16K in the F_2 dimension. Suppression of t_1 ridges was achieved by multiplying the first row of the (t_1, ω_2) data matrix by 0.5. Baseline correction was performed by applying a fifth-order polynomial in both dimensions following Fourier transformation.

RESULTS

Circular Dichroism Studies

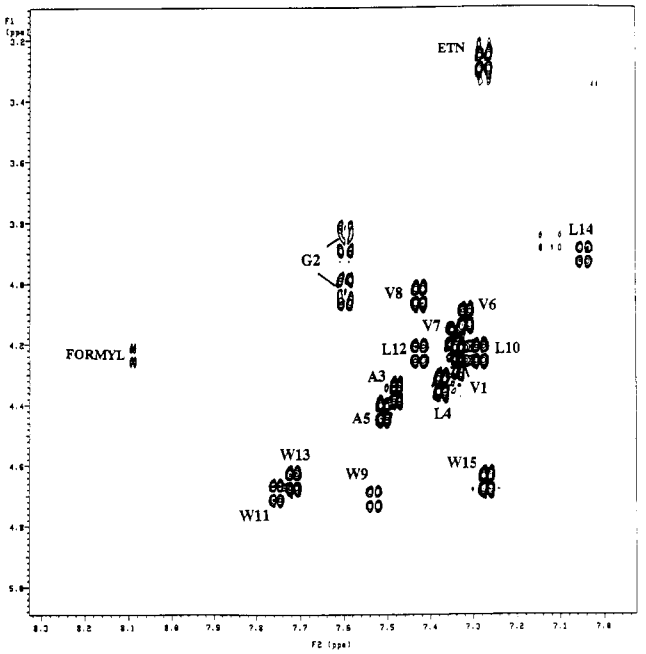
The environmental transitions along the pathway to the formation of gramicidin A channels can be summarized as

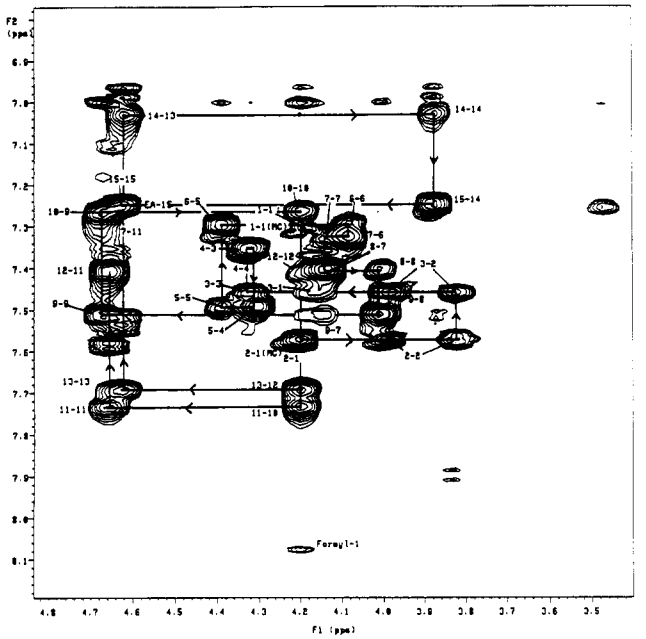
$$\underset{\text{clear soln}}{\text{neat TFE}} \rightarrow \underset{\text{sonication}}{\text{TFE/water/micelles}} \xrightarrow{\text{sonication}} \underset{\text{clear}}{\text{micelles}}$$

Figure 1 shows the dramatic differences in the CD spectra for gramicidin A obtained in TFE, 10% TFE, and SDS micelles, clearly indicating that gramicidin A undergoes a conformational transition during incorporation to the channel state in SDS micelles. The CD spectrum of gramicidin A in SDS micelles is characteristic of the single-stranded $\beta^{6.3}$ helical channel state (Masotti et al., 1980; Wallace et al., 1981). The CD spectrum of gramicidin A in TFE is characterized by a weak positive ellipticity around 222 nm, similar to that obtained by Heitz and Spach (1975) for the synthetic L,D-peptides, poly(benzyl L,D-glutamate), in TFE. An X-ray diffraction technique was used to confirm that the conformation that corresponds to this type of CD pattern for L,D-peptides was the α-helix (Heitz & Spach, 1975).

Proceeding from the environment of TFE to 10% TFE, the CD spectrum of gramicidin A shows a negative ellipticity around 230 nm and a strong positive ellipticity around 197 nm resembling the CD spectra for the peptide obtained in alcohol solutions and ethyl acetate (Wallace et al., 1986; Veatch et al., 1974). In these solvents the peptide exists as a mixture of four different forms of intertwined double-stranded helices, i.e., a symmetric left-handed parallel $\beta^{5.6}$ double helix, an unsymmetric left-handed parallel $\beta^{5.6}$ double helix (which differs from the latter in the "stagger" of the polypeptide chains), a symmetric left-handed antiparallel $\beta^{5.6}$ double helix, and a symmetric right-handed parallel $\beta^{5.6}$ double helix (Veatch et al., 1974; Bystrov & Arseniev, 1988). On the basis of these apparent similarities, it is very likely that the conformational state of gramicidin A in 10% TFE may be similar to that found in alcohols and ethyl acetate. The minor differences (that is, magnitude and small shifts in ellipticities around 198, 219, and 230 nm) observed between the CD spectra in these solvents may be attributed to the variation in the relative populations of the conformers and/or the existence of different forms of double-helical species.

Previous studies have shown that the conformation of gramicidin A varies with the organic solvent environment, peptide concentration, and temperature. Perhaps due to the hydrophobicity and insolubility of gramicidin A in water (<10 mg/L) (Wallace, 1990) there has not been much data on the conformation of gramicidin A in aqueous or a mixture of aqueous–organic solution. Hence, CD studies of gramicidin A in varying ratios of TFE/water were undertaken to investigate the effect on gramicidin A conformation with increasing water concentration. Figure 2 shows CD spectra of 100 μM gramicidin A obtained at 15%, 30%, 55%, and 100% TFE concentration. As can be seen, with increasing water concentration the CD spectra undergo a change toward that characteristic of the mixture of double-stranded β-helices (appearance of ellipticities at 198, 219, and 230 nm). In addition, consistent with previous observations on the concentration dependence of gramicidin A conformation, the formation of double-helical species in an aqueous environment in the present study was also found to be dependent upon peptide concentration. It was observed that at 100 μM gramicidin A concentration the change in the CD curve characteristic of the double helix began at 30% TFE whereas





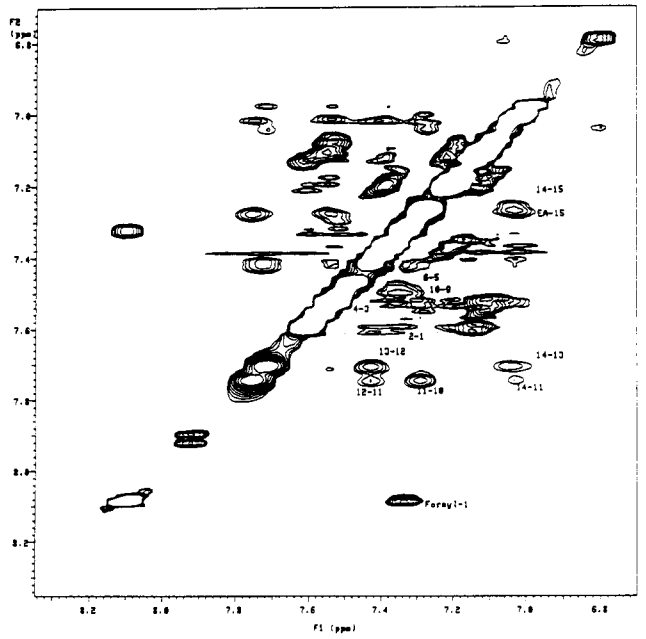


Table 1: ^{1}H NMR Chemical Shifts (in ppm Relative to Internal Tetramethylsilane) of Gramicidin A in TFE at 25 °C

residue	NH	CαH	CβH	others	$^3J_{HN\alpha}$
formyl				HCO 8.11	
L-Val 1	7.32	4.24	1.89	γCH$_3$ 1.01	7.02
Gly 2	7.58	4.00, 3.86			
L-Ala 3	7.45	4.34	1.39		5.18
D-Leu 4	7.35	4.31	1.59	γCH 1.67; δCH$_3$ 0.92	6.43
L-Ala 5	7.48	4.40	1.37		6.44
D-Val 6	7.29	4.10	2.12	γCH$_3$ 0.90	7.53
L-Val 7	7.31	4.18	2.12	γCH$_3$ 0.98	overlap
D-Val 8	7.40	4.02	2.12	γCH$_3$ 0.83	7.46
L-Trp 9	7.51	4.69	3.28		6.76
D-Leu 10	7.26	4.22	1.22	γCH 1.45; δCH$_3$ 0.56, 0.67	7.31
L-Trp 11	7.22	4.66	3.33		6.15
D-Leu 12	7.40	4.22	1.21	γCH 1.39; δCH$_3$ 0.62	7.46
L-Trp 13	7.70	4.62	3.25, 3.27		6.68
D-Leu 14		3.91	1.02	γCH 1.21; δCH$_3$ 0.55	7.46
L-Trp 15	7.26	4.63	3.18, 3.36		7.32
EA	7.26	3.27	3.49		

at 50 μM concentration this change was observed at 20% TFE (data not shown).

NMR Spectroscopy

Assignment of the 2D NMR spectra of gramicidin A in the different solvent environments was accomplished using the sequential assignment technique developed by Wüthrich (1986). This involved the identification of the spin systems using DQF-COSY, relayed-COSY, and HOHAHA (at mixing times of 50, 65, 75, and 80 ms), followed by sequential assignment using NOESY. Secondary structure analysis involved identifying NOE cross-peaks between protons on nonneighboring residues and measuring $^3J_{HN\alpha}$ coupling constants from the antiphase NH–CαH cross-peaks in the DQF-COSY spectrum as well as comparisons of αH chemical shifts. For spectra with multiple conformers, conformational analysis was aided by comparisons with spectra from previous work (Bystrov et al., 1988).

Gramicidin A in Neat TFE. The DQF-COSY spectrum of the "fingerprint" region of gramicidin A in TFE (Figure 3a) showed a total of 17 cross-peaks arising from the 15 amino acid residues, the formyl group, and the ethanolamine group. When the vertical scale was extended close to the noise level, several very weak cross-peaks were observed very close in proximity to the formyl cross-peak. These weaker cross-peaks are believed to be the formyl groups of some minor conformers in fast interconversion with the major gramicidin conformer. The fact that cross-peaks belonging to minor conformers were not observed for other gramicidin residues may be due to slower molecular motion at the NH_2 terminus, resulting in nondegenerate formyl cross-peaks corresponding to the different conformers. Table 1 gives a summary of ^{1}H chemical shift assignments and $^3J_{HN\alpha}$ coupling constants for gramicidin A in TFE.

Figure 3b shows the fingerprint region of the NOESY spectrum and the sequential connectivities of the backbone protons. The sequential NOE cross-peaks, $d_{\alpha N}$ (αH_i–NH_{i+1}), and the d_{NN}(NH_i–NH_{i+1}) NOE connectivities (Figure 3c) are poorly dispersed with many overlaps, suggestive of a flexible monomeric structure (Roux et al., 1990; Hawkes et al., 1988). The observation of two nonneighboring NOE interactions,

FIGURE 3: Expanded regions of (a, top) NH–αH of the phase-sensitive DQF-COSY spectrum, (b, middle) NH–αH of the phase-sensitive NOESY (200-ms mixing time), and (c, bottom) NH–NH of NOESY for 53 mM gramicidin A in TFE. The spectra were recorded at 25 °C.

the NH(Ala^3)–CαH(Val^7) and NH(Trp^{11})–NH(Leu^{14}), is indicative of a folded region. A ROESY experiment was also carried out to detect the presence of additional NOEs diagnostic of a folded structure that may not have been detected in NOESY spectra due to unfavorable correlation time, τ_c (Dyson & Wright, 1991). Upon analysis of the fingerprint region of the ROESY spectrum, three cross-peaks due to $d_{N\alpha}(i,i+4)$ and $d_{N\alpha}(i,i+2)$ interactions were observed. These NOE interactions involved NH(Ala^3) and CαH(Val^7), NH(Ala^5) and CαH(Trp^9), and NH(Val^7) and CαH(Trp^9). The observation of two additional NOEs, NH(Ala^5)–CαH(Trp^9) and NH(Val^7)–CαH(Trp^9), not observed in NOESY is in support of the folded conformation as well.

Gramicidin A in 55% TFE. The conformation of gramicidin A in an aqueous environment is of interest since it represents the transient state before channel formation in micelles. Since gramicidin A spontaneously precipitates below 55% TFE concentration, NMR studies were carried out in this solvent mixture in order to characterize the conformation of gramicidin A in the presence of the highest concentration of water before precipitate formation. The fingerprint region of the DQF-COSY spectrum (Figure 4a) is similar to that for gramicidin A in TFE in terms of poor cross-peak dispersion and the general location of NH–CαH cross-peaks. The biggest difference between the spectra for gramicidin A in 55% TFE and those in neat TFE is the presence of more than one set of very weak cross-peaks besides the major ones. These weak cross-peaks have been assigned to minor conformers.

As seen in Figure 4b, nonneighboring intramolecular NOEs detected in this system include two $d_{\alpha N}(i,i+2)$ interactions for CαH(Leu^4)–NH(Val^6) and CαH(Val^8)–NH(Leu^{10}) and one $d_{\alpha N}(i,i+4)$ interaction between CαH(Leu^{12}) and CαH-(Leu^{12}), as well as an interaction between the CβH side-chain protons of Trp^{11} and Val^8. As in the case of gramicidin in TFE, a complete set of d_{NN} connectivities of medium intensity was observed. Table 2 shows a summary of the proton chemical shifts and $^3J_{NHC\alpha}$ values, both of which are similar to those observed for gramicidin A in TFE. Taken together, the NOEs, $^3J_{NHC\alpha}$, and CD data suggest the presence of secondary structure for gramicidin A in 55% TFE very similar to that obtained in neat TFE. A completely random coil state, on the other hand, would give NMR spectra devoid of any nonneighboring NOEs as well as only one set of cross-peaks representing the conformationally averaged structure.

Gramicidin A in 10% TFE/Dioxane. Since gramicidin A in 10% TFE is insoluble, conformational studies were carried out by isolating the precipitate formed in 10% TFE and dissolving it in dioxane in which gramicidin conformers undergo very slow interconversion. The DQF-COSY spectrum of gramicidin A in 10% TFE/dioxane is shown in Figure 5a. For labeling convenience, letter codes A, B, C, etc. are used to refer to the different polypeptide chains while the amino acid residues are referred to by numbers corresponding to their positions in the sequence. At least 150 intraresidue NH–CαH cross-peaks were observed in the fingerprint region of the DQF-COSY spectrum, suggesting the coexistence of at least seven different gramicidin A conformers. The existence of multiple resonance lines due to the different species results in many degeneracies and resonance overlaps. Though difficult, assignment of most of the spin systems was possible using DQF-COSY and especially HOHAHA experiments recorded at mixing times of 50, 65, and 75 ms. After spin system assignments were made, a comparison with the previously published NMR spectra of double-helical species (Bystrov & Arseniev, 1988) was made to identify these species on the basis of cross-peak patterns. These identified species

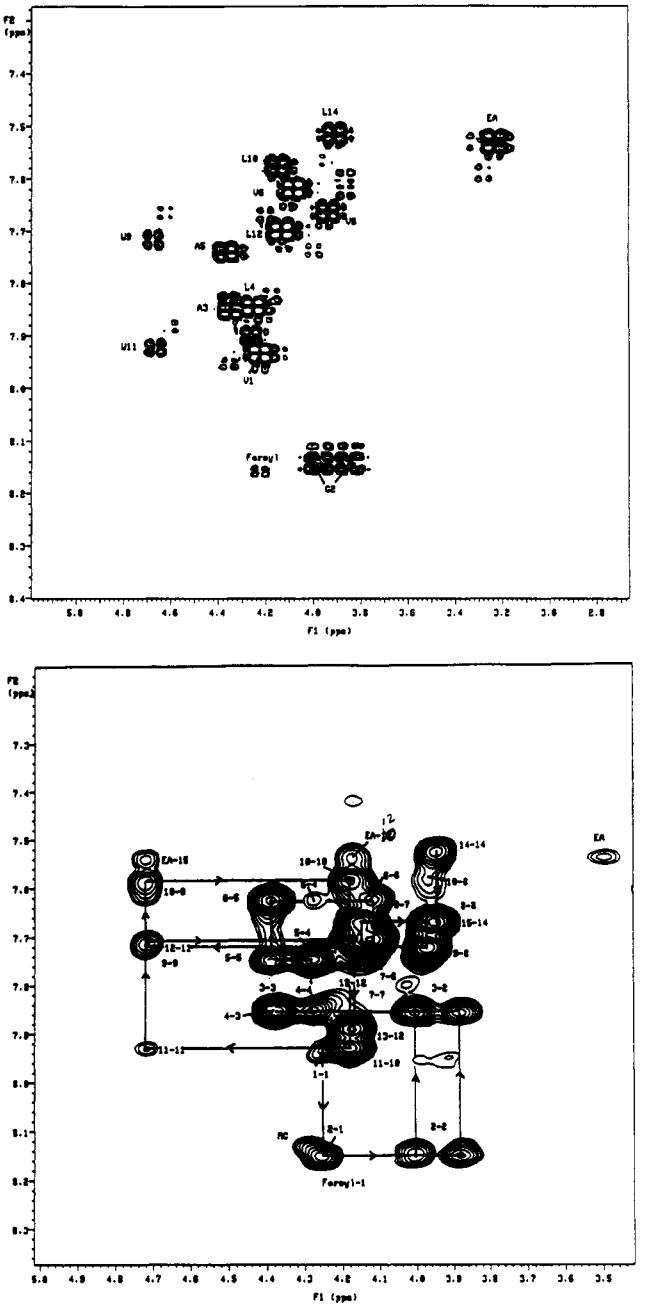


FIGURE 4: Expanded regions of (a, top) NH–αH of the phase-sensitive DQF-COSY and (b, bottom) NH–αH of the phase-sensitive NOESY (200-ms mixing time) for 7 mM gramicidin A in 55% TFE. Two $d_{\alpha N}(i,i+2)$ NOEs for NH(Val^6)–CαH(Leu^4) and NH(Leu^{10})–CαH-(Val^8) and one $d_{\alpha N}(i,i+4)$ interaction between NH(EA) and CαH-(Leu^{12}) are shown. The spectra were recorded at 25 °C. The NH–αH cross-peaks for Trp^{15} and Trp^9 are not observed since the α-protons coincide with the water resonance. The weak cross-peaks that appear around the strong cross-peaks correspond to minor conformations.

are as follows: a symmetric left-handed parallel $\beta^{5,6}$ double helix, an unsymmetric left-handed parallel $\beta^{5,6}$ double helix, a symmetric left-handed antiparallel $\beta^{5,6}$ double helix, and a symmetric right-handed parallel $\beta^{5,6}$ double helix. These conformations are referred to as the double helices A, B–C, D, and E by Bystrov and Arseniev (1988) which correspond to species 1, 2, 3, and 4 of Veatch et al. (1974). Evidence for the presence of a symmetric right-handed antiparallel $\beta^{5,6}$ double helix was also found in the DQF-COSY spectrum. However, weak NOESY and overlapped DQF-COSY peaks made this identification less certain than for the other double helices.

The cluster of cross-peaks in the region between 7.7 and 8.3 ppm and between 4.0 and 4.9 ppm in the F_2 and F_1 dimensions,

Table 2: ^{1}H NMR Chemical Shifts (in ppm Relative to Internal Tetramethylsilane) of Gramicidin A in 55% TFE at 25 °C

residue	NH	CαH	CβH	others	$^3J_{HN\alpha}$
formyl				HCO 8.16	
L-Val 1	7.94	4.23	2.17	γCH$_3$ 1.01	6.71
Gly 2	8.15	3.89, 4.00			
L-Ala 3	7.86	4.35	1.42		5.90
D-Leu 4	7.85	4.25	1.42, 1.64	γCH 1.64; δCH$_3$ 0.90	6.72
L-Ala 5	7.75	4.35	1.39		6.72
D-Val 6	7.62	4.10	2.10	γCH$_3$ 0.91	6.56
L-Val 7	7.71	4.14	2.15	γCH$_3$ 0.94	5.92
D-Val 8	7.67	3.97	2.05	γCH$_3$ 0.75	6.7
L-Trp 9	7.73	4.68	3.18, 3.30		
D-Leu 10	7.58	4.16	1.17	γCH 1.36; δCH$_3$ 0.51	5.9
L-Trp 11	7.93	4.72	3.28, 3.31		
D-Leu 12	7.52	3.94	1.01	γCH 1.14; δCH$_3$ 0.51	5.9
L-Trp 13	7.88	4.64	3.12, 3.38		
D-Leu 14	7.71	4.25	1.19	γCH 1.32; δCH$_3$ 0.49	6.22
L-Trp 15	7.68	4.64	3.15, 3.35		
EA	7.53	3.26	3.28		

respectively, has been assigned to flexible, monomeric species on the basis of the following observations: (1) The cross-peaks assigned to this monomeric species (see corresponding region in Figure 5a) are poorly dispersed and consist of more or less 15 cross-peaks (the uncertain count is due to cross-peak overlaps) identified as valines, leucines, alanines, and tryptophans. (2) The amino acid composition is consistent with that expected for gramicidin A. (3) Both the NH and αH chemical shifts of the amino acids are very similar to those expected for a flexible, monomeric gramicidin A.

Due to problems with cross-peak overlap and low signal-to-noise ratios of the less populated species, as well as nonobservable NOEs due to unfavorable correlation time, complete unambiguous assignments of the remaining conformers could not be made. Clearly, the best way to identify all the conformers present would be to isolate the different species and analyze them individually. Nonetheless, on the basis of the observation that the cross-peaks are very well dispersed, it is very likely that the remaining unassigned resonances belong to intertwined double-helical species which are different from those identified, perhaps in the stagger of their polypeptide chains, number of residues per helical turn, and handedness.

From the cross-peak intensities a qualitative approximation could be made on the relative population of the different species that exist in the system. The symmetrical left-handed antiparallel $\beta^{5,6}$ double helix (species 3), the most abundant of all the conformers, makes up approximately 35% of the conformational population. Each of the remaining species constitutes approximately 15% of the population or less. The representative ^{1}H chemical shift assignments and values of $^3J_{HNC\alpha}$ coupling constants for species 3 are summarized in Table 3.

Gramicidin A in 10% DMSO/Dioxane and 10% Methanol/Dioxane. The effect of solvent on the conformational transition of gramicidin during the channel formation process has also been investigated. It was, therefore, not surprising to see that the DQF-COSY spectrum for gramicidin A in 10% DMSO/dioxane (Figure 6a) was considerably different from the corresponding spectrum for gramicidin A in 10% TFE/dioxane. The dominant feature in the DQF-COSY spectrum shown in Figure 6a is the intense cross-peaks that are grouped together in a region between 7.8 and 8.3 ppm and between 3.8 and 4.7 ppm in the F_2 and F_1 dimensions, respectively. In addition, cross-peaks of much weaker intensities which are more dispersed (over an area of approximately 1.5 ppm in both F_1 and F_2 dimensions) are observed. Following the sequential assignment methods the group of intense cross-peaks which constituted approximately 70% of the conformational population has been assigned to a monomeric species which is very likely to be random coil. On the basis of their cross-peak patterns, the weaker cross-peaks were assigned to the intertwined double-helical species similar to those observed in the 10% TFE/dioxane system. Besides demonstrating the solvent dependence of the conformational states, this study serves as a control to demonstrate the ability of dioxane in preserving the nonequilibrium conformation of gramicidin A.

The 2D NMR spectrum obtained for the gramicidin A precipitate isolated from 10% methanol was very similar to that obtained in neat methanol (data not shown). Both spectra exhibit cross-peak patterns characteristic of the conformational mixture of the four double-helical species A, B–C, D, and E obtained previously in ethanol (Bystrov & Arseniev, 1988).

DISCUSSION

While many studies of gramicidin A have been directed toward the understanding of its structural and ion channel properties, only very few have addressed the problem involving the mechanism of ion channel insertion/formation in membranes (O'Connell et al., 1990; Zhang et al., 1992). Since it has been observed that gramicidin A solubilized in an organic solvent spontaneously precipitates upon addition to aqueous SDS micelle solution, the present study has addressed the question of the channel formation pathway by undertaking conformation studies of gramicidin A in organic and organic–aqueous environments. Besides determining the conformation of gramicidin A in TFE, more importantly this study differs from previous attempts in addressing the channel formation problem in that the conformational state of gramicidin A in the transient aqueous environment has been characterized.

The conformational and environmental transitions along the pathway to the formation of gramicidin A channels can be summarized as

$$\underset{\text{monomer}}{\text{neat TFE}} \rightarrow \underset{\text{>85\% double helices}}{\text{TFE/water/micelles}} \xrightarrow{\text{sonication}} \underset{\text{channel form}}{\text{micelles}}$$

Results from 2D NMR and CD studies presented earlier suggest that gramicidin A in TFE exists as a monomer. Minor conformers in fast interconversion with the major form are also believed to be present on the basis of the two following experimental observations: (1) Although a single set of cross-peaks was present for gramicidin A amino acid residues, several overlapping formyl-Val^1 cross-peaks were observed in the fingerprint region. This observation is very likely due to slower molecular motion at the CH_2 terminus resulting in nondegenerate formyl cross-peaks for the different conformers. (2) The net CD spectra of gramicidin A in neat TFE and in 55% TFE are similar, but the NMR spectra of the peptide in 55% TFE show several sets of intraresidue, $d_{\alpha N}$ COSY cross-peaks, suggesting that the different monomeric conformers present in 55% TFE are also present in neat TFE but conformational interconversion in the former is relatively slower than the latter on the NMR time scale.

The presence of the medium-range NOEs [$d_{N\alpha}(i,i+4)$ and $d_{N\alpha}(i,i+2)$] and the measured values of $^3J_{NHC\alpha}$ coupling constants provide evidence for the presence of secondary structure for gramicidin A in TFE and in 55% TFE. As shown in Tables 1 and 2, the measured $^3J_{NHC\alpha}$ coupling constants are in the range of 5.2–7.5 Hz. While it may be argued that this range of $^3J_{NHC\alpha}$ values may suggest a random coil structure, a better explanation which would be consistent with the NOE data would be in terms of conformational averaging between two conformations, the L,D-$\alpha^{3,6}$ and $\beta^{4,4}$, which have

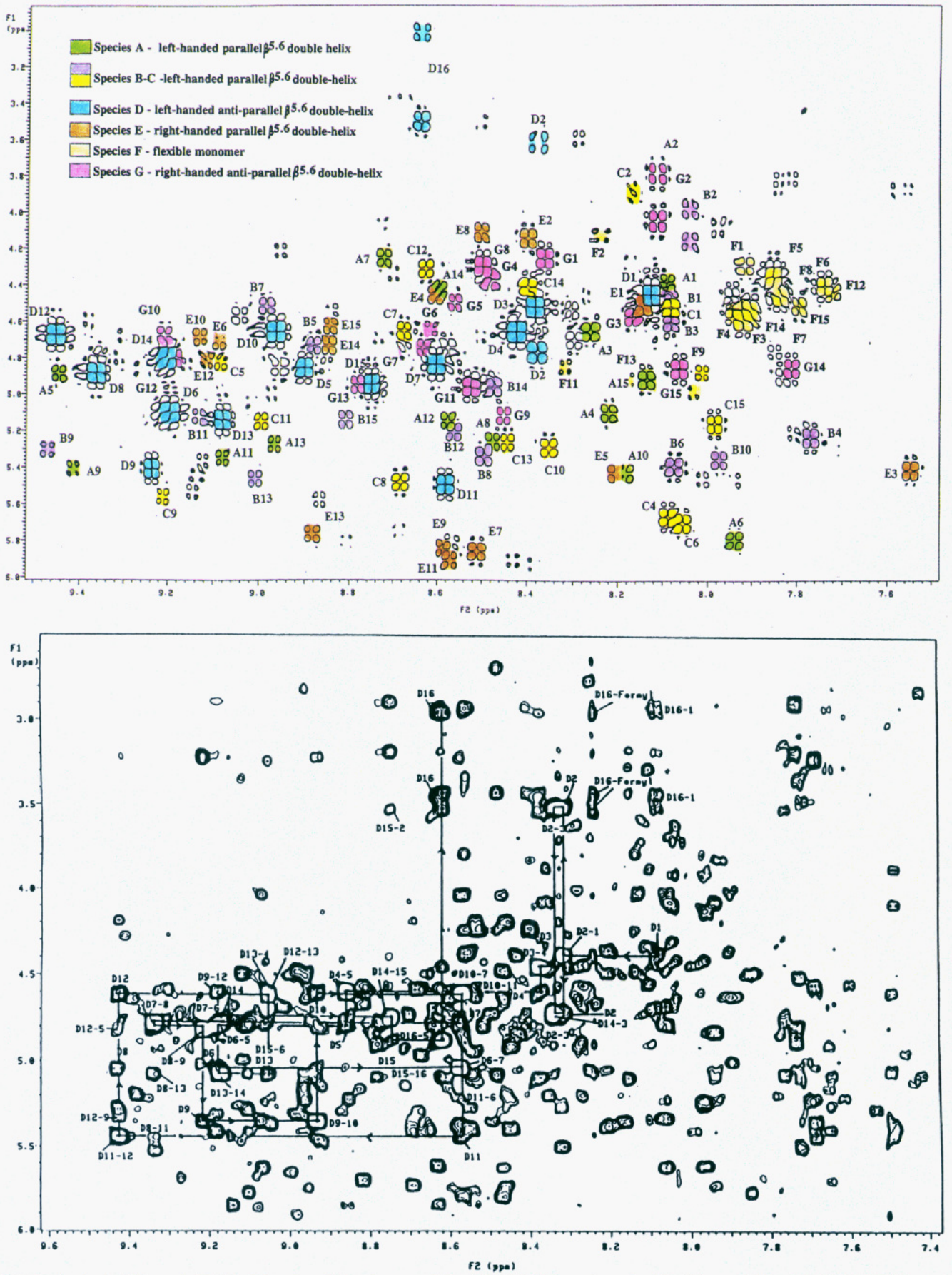


FIGURE 5: (a, top) Expanded NH–αH region of the phase-sensitive DQF-COSY spectrum for 10 mM gramicidin A in 10% TFE/dioxane at 25 °C. The capital letters (A, B, C, D, etc.) preceding the residue number refer to polypeptide chains corresponding to the different conformers. Note that species 2 is the only unsymmetrical conformer and exhibits two sets of cross-peaks (labeled as B and C) corresponding to two polypeptide chains that make up the double-stranded $\beta^{5.6}$ helical structure. The other conformers which are symmetrical exhibit one set of degenerate resonances for both polypeptide chains that make up the double helix. (b, bottom) Expanded NH–αH region of the phase-sensitive NOESY (200-ms mixing time) for the same sample at 25 °C. The $d_{\alpha N}$ sequential connectivities for the major conformer (species 3, residues labeled with the letter D) as well as nonneighboring $d_{\alpha N}$ NOEs are labeled.

Table 3: ^{1}H NMR Chemical Shifts (in ppm Relative to Internal Tetramethylsilane) of Peptide Chain D (Species 3) for Gramicidin A in 10% TFE at 25 °C

residue	NH	CαH	CβH	others	$^3J_{HN\alpha}$
L-Val 1	8.13	4.44	2.09	γCH$_3$ 1.01	8.65
Gly 2	8.38	4.76, 3.59			
L-Ala 3	8.38	4.50	1.21		7.78
D-Leu 4	8.42	4.65	1.41, 1.43	γCH 1.28; δCH$_3$ 0.71, 0.50	
L-Ala 5	8.90	4.84	1.08		8.03
D-Val 6	9.20	5.08	2.21	γCH$_3$ 1.00	10.03
L-Val 7	8.60	4.83	2.14	γCH$_3$ 0.91, 0.83	8.12
D-Val 8	9.36	4.86	2.17	γCH$_3$ 1.18	8.84
L-Trp 9	9.23	5.42		overlap	7.64
D-Leu 10	8.97	4.64	0.80, 0.94	γCH 1.33; δCH$_3$ 0.56, 0.33	9.00
L-Trp 11	8.58	5.49	3.18, 3.05		8.58
D-Leu 12	9.45	4.66	1.53	γCH 1.37; δCH$_3$ 0.81, 0.64	8.73
L-Trp 13	9.08	5.12	3.18, 2.97		7.72
D-Leu 14	9.21	4.79	1.30, 1.17	γCH 0.94; δCH$_3$ 0.56, 0.26	9.32
L-Trp 15	8.75	4.93	3.23, 2.95		7.93
EA	8.64	3.58, 3.02	3.48		

been predicted to be energetically favorable (Colonna-Cesari et al., 1977; Hesselink & Scheraga, 1972; Ramachandran & Chandrasekaran, 1972). In fact, the average value of the theoretical $^3J_{NHC\alpha}$ coupling constant for the α helix (4–6 Hz) and $\beta^{4,4}$ helix (7.5–10 Hz) (DeMarco et al., 1978) for L,D-peptides closely approximates the measured $^3J_{NHC\alpha}$ values of 5.2–7.5 Hz for gramicidin A in TFE and in 55% TFE. Furthermore, as indicated earlier the similarity between the CD spectra of gramicidin A in TFE and 55% TFE to that obtained for the α-helical, poly(benzyl L,D-glutamate) lends further support on the presence of the α-helical conformation. On the basis of the conformational energy calculations, both left- and right-handed helices are equally favored for peptides consisting of L- and D-amino acid residues. However, the appearance of the CD spectra (weak positive ellipticity around 222 nm) may suggest a preference for one type of handedness since an equal population of right- and left-handed conformers would give a net CD absorption close to zero.

Gramicidin A in 10% TFE/water was found to consist of a mixture of at least seven different conformations based on the number of intraresidue DQF-COSY cross-peaks. Comparisons with previous 2D NMR spectra (Bystrov & Arseniev, 1988) revealed that four of these conformations were similar to the intertwined double-helical species which have been identified previously as species 1, 2, 3, and 4 (Veatch et al., 1974; Bystrov & Arseniev, 1988). On the basis of chemical shift data (very well dispersed cross-peaks) and $^3J_{NHC\alpha}$ coupling values, it is believed that the remaining species present in the conformation mixture include monomeric species and other forms of intertwined double helices.

2D NMR studies of gramicidin A in 10% DMSO revealed that approximately 70% of the conformational population in a monomer while the remainder consists of intertwined double-helical species. The 2D NMR spectra obtained for gramicidin A in 10% methanol and in neat methanol are almost identical. These spectra in turn resembled the spectrum obtained for gramicidin A in ethanol, suggesting a similar conformational state [i.e., consisting of the mixture of species 1, 2, 3, and 4 (Bystrov & Arseniev, 1988)] for gramicidin A in all three solutions.

By extrapolation of these conformational data to the channel formation process, it can be concluded that the nature of the folding *intermediates* (that is, the conformation of gramicidin A in the aqueous environment) is dependent upon the solvent history. As has been demonstrated in the case for gramicidin A in TFE, these *intermediates* may not be the same as the conformation adopted in the starting organic solvent. Hence, the ease of channel incorporation is dependent on the conformation of the *intermediates* in the aqueous environment rather than the *starting* structure of gramicidin A in organic solvent as has been previously thought. Furthermore, the presence of several conformational intermediates suggests that more than one conformational pathway is possible leading to the thermodynamically stable channel state.

On the basis of the present results the molecular mechanism involved in gramicidin channel formation in SDS micelles or lipid bilayers using TFE as the starting solvent can be described as follows. In TFE, gramicidin A is a monomer that consists of a major and several minor helical conformers in fast interconversion. Upon addition of gramicidin (from TFE) to the aqueous micelle solution a spontaneous precipitation occurs. The precipitated gramicidin or the *intermediates* consist of a mixture of different forms of intertwined double helices as well as monomeric form(s). The nature of these *intermediates* suggests that one pathway to the channel state involves gramicidin monomers. Channels formed via gramicidin monomers would follow a similar mechanism described previously (O'Connell et al., 1990) where the monomers are initially adsorbed onto the lipid bilayers or micelles and then inserted and assembled into the single-stranded helical dimer channel. Another pathway to the channel state is by the rearrangement of the intertwined antiparallel helical forms described previously (Zhang et al., 1992). Channel formation from parallel intertwined helical species is more difficult since it requires an initial unwinding/disassociation of the intertwined helices to monomers, followed by the assembly into the helical dimer channel. Thus, the unwinding of parallel intertwined species would appear to be the rate-limiting step in channel formation. As the gramicidin monomers and double helices in solution are being incorporated into micelles, dissolution of the precipitate takes place concomitantly. Sonication provides the energy needed in the disassociation/unwinding process of the intertwined parallel dimer species to monomers.

The observed solvent dependence on the ease of channel formation appears to be related to the nature of the dimer forms in the aqueous environment as has been discussed previously (LoGrasso et al., 1988; Wallace, 1990). In addition, based on previous results (Masotti et al., 1980; Tournois et al., 1987) and results from the present studies, it appears that organic solvents that provide an environment favorable for gramicidin monomers are also important in channel incorporation. This is especially crucial in the case of incorporating a high concentration of gramicidin A into SDS micelles for NMR structural studies. When solvents other than TFE such as methanol or ethanol were used, the precipitate formed in the aqueous micelle solution persisted even after prolonged sonication. Studies on the incorporation of gramicidin channels into lipid bilayers are also in support of this. It was shown that, by addition of gramicidin solubilized in either TFE or DMSO, channels were incorporated directly in lipid bilayers without incubation and/or sonication (Killian et al., 1988). The fact that solvent dependence was not observed in the case of single channel conductance studies (Sawyer et al., 1990) may be because the concentration of gramicidin used in the conductance studies is so low that it exists mostly as monomers which form channels readily.

From a broader perspective, the conformational transition along the pathway to channel formation demonstrates an example of protein folding events which involve the interplay

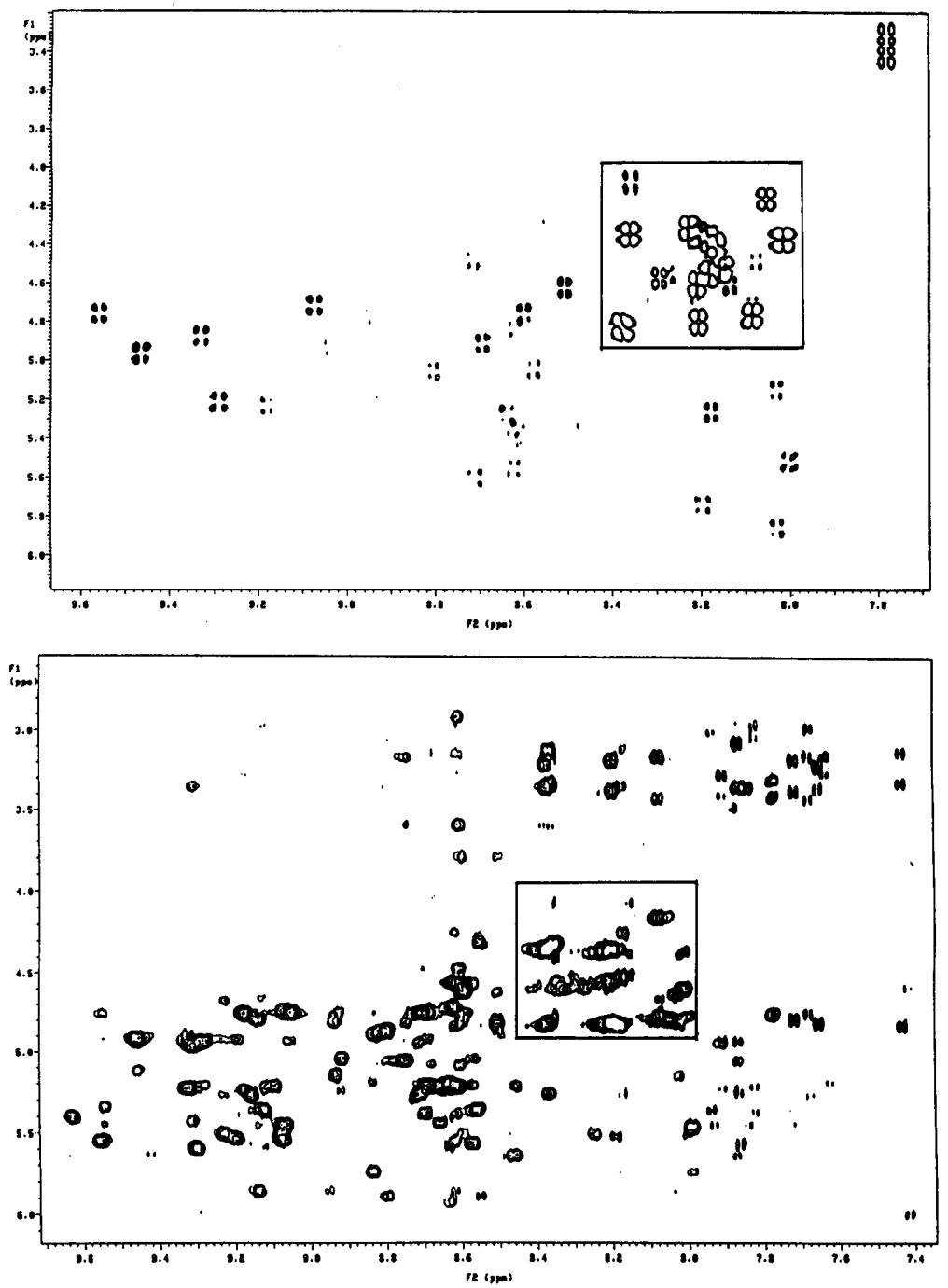


FIGURE 6: Expanded regions of (a, top) NH–αH of the phase-sensitive DQF-COSY spectrum and (b, bottom) NH–αH of the phase-sensitive NOESY (200-ms mixing time) for 10 mM gramicidin A in 10% DMSO/dioxane. Cross-peaks enclosed in the boxed region (7.8–8.3 and 3.8–4.7 ppm in the F_2 and F_1 dimensions, respectively) correspond to the major random coil conformer. The spectra were recorded at 25 °C.

between the amino acid sequence and the environment as well as between kinetic and thermodynamic folding products (Jaenicke, 1991). In addition, interactions such as electrostatics, van der Waals, hydrogen bonding, hydrophobic, and intrinsic propensities are the driving forces in protein folding (Dill, 1990). There is now strong evidence that hydrophobicity is the dominant force in protein folding (Dill, 1990). It appears that for gramicidin A its amino acid sequence specifies its intrinsic propensities (i.e., hydrophobicity and membrane affinity) while the environment is the dominant factor in deciding its final folded state. Moreover, hydrophobicity and hydrogen-bonding interactions appear to be involved in concert in defining the final folded state of gramicidin A molecules in a given environment. In nonpolar environments, such as dioxane or membranes, inter- and intramolecular hydrogen bonds appear to be the dominant structure-determining interactions for gramicidin A. In these environments gramicidin A forms the double-stranded, intertwined helices and ion channel structures, respectively. Both structures are stabilized by extensive hydrogen bonding that maximizes hydrophobic interactions between gramicidin molecules or between gramicidin and lipids. That the single-stranded, $\beta^{6.3}$ helical dimer channel structure is the most thermodynamically stable, ion transporting state in membranes is due to the following factors. The first is that the $\beta^{6.3}$ helical dimer spans the lipid bilayer almosot in its entirety, thereby maximizing hydrophobic interactions between gramicidin A and lipid molecules, and second, additional stability of the helical dimer channel is provided by hydrogen bonding of the indole NH groups to the surface of the lipid bilayer (O'Connell et al., 1990; Scarlatta 1991; Durkin et al., 1992).

As the polarity of the organic solvent increases, the interaction between the solute molecules (i.e., gramicidin A) decreases due to the ability of the polar solvent molecules to compete for hydrogen bonding with the solute molecules. This is corroborated by the observation of an increase in the rate of interconversion between the various intertwined helical structures in more polar organic solvents. For example, gramicidin A dissolved in dioxane retains its original conformation(s) for periods exceeding 2 weeks, whereas in ethanol the rate of interconversion is significantly faster (less than 15 h) (Veatch & Blout, 1974; Pascal & Cross, 1992). The latter can be explained by the better ability of ethanol than dioxane to compete for hydrogen-bonding sites. Assuming that the dielectric constant is a crude measure of the polar nature of a solvent, one would predict more monomers in methanol (dielectric constant = 32.6) than in ethanol (dielectric constant = 24.3). Evidence for this was found in fluorescence measurements which indicated that about 20% of the gramicidin A population in methanol at a concentration of 5×10^{-2} M was monomeric while in ethanol only 2% of the peptide population was a monomer (Fossel et al., 1974). In DMSO (dielectric constant = 46.7) and TFE the percentage of monomeric population approaches 100%, implying a complete disruption of the solute intermolecular hydrogen bonds by these more polar solvents. These observations suggest that solute–solvent interaction is another important driving force in the folding of gramicidin A. The solute–solvent interactions are provided by hydrogen-bonding as well as hydrophobic interactions between the solvent molecules (which also have hydrophobic characteristics as evidenced by their ability to solvate high concentrations of gramicidin) and the nonpolar hydrophobic regions of gramicidin molecules.

The fact that gramicidin A favors the dimeric forms with decreasing ratio of TFE/water may appear to contradict the above discussion on its preference for monomeric forms in highly polar solvents. The phenomenon observed in the aqueous environment can be explained in terms gramicidin's intrinsic hydrophobicity being the dominant driving force of folding in water. Hence, in a fast kinetic process, nonpolar hydrophobic gramicidin molecules associate with one another via hydrogen bonding in conformations that offer the smallest surface area possible to water molecules. That this type of conformation is highly favorable in an aqueous environment is due to the fact that the free energy of stabilization of a folded protein is proportional to the water-accessible surface area (Richards, 1977).

However, for gramicidin A in the DMSO/water system the major population (approximately 70%) is a monomer compared to predominantly dimer forms in the TFE/water system. Although other explanations are certainly possible, this apparent paradox to what was observed in the TFE/water system can be explained in terms of the greater solubilizing effect of DMSO (being more polar than TFE) as well as the slower mixing of DMSO with water due to its relatively high viscosity. It is believed that strong solvation spheres formed around gramicidin A molecules by DMSO (due to hydrophobic interactions) together with its relatively slow mixing with water result in the random coil conformation being the major kinetic product in the spontaneous precipitation process. The formation of these disparate intermediate species in a given environment can be rationalized if one considers the intricate balance between stabilizing and destabilizing forces in defining the peptide's final structure and the small difference in the free energy of stabilization between the different conformations (Dill, 1990).

In summary, the present investigation represents the first effort to characterize the conformational *intermediates* of gramicidin A prior to insertion and ion channel formation using NMR and CD techniques. On the basis of the results from these studies, the observed dependence of the ease of channel formation on solvent history can be attributed to the nature of conformational intermediates in the organic–aqueous environment. Hence, the solvent environment as opposed to the intrinsic property of gramicidin A (i.e., its amino acid sequence) is the dominant driving force in the folding of gramicidin A. The channel form of gramicidin A is an accurate representation of a thermodynamically stable folded state since its achievement does not depend on the initial condition or the pathway/process leading to this state as demonstrated by the presence of several conformational pathways to the channel form.

SUPPLEMENTARY MATERIAL AVAILABLE

Expanded regions from the NMR spectra of the d_{NN} region from the NOESY spectrum of gramicidin A in 55% TFE, the $d_{\alpha N}$ region of ROESY for gramicidin A in neat TFE, and the $d_{\alpha\alpha}$ and d_{NN} regions from the NOESY spectrum of gramicidin A in 10% TFE and tables summarizing ^{1}H NMR assignments, chemical shifts, and $^3J_{NHC\alpha}$ values for the different conformers (other than species 3) of gramicidin A in 10% TFE (10 pages). Ordering information is given on any current masthead page.

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