JOURNAL OF MAGNETIC RESONANCE **124,** 132–139 (1997)
ARTICLE NO. MN967493

Effects of Alanine and Glycine Substitution for Tryptophan on the Heterogeneity of Gramicidin A Analogs in Micelles

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Received September 25, 1996

The effects of alanine and glycine substitution for tryptophan upon the species heterogeneity of gramicidin A analogs incorporated into SDS micelles have been investigated. The sequential replacement of the four tryptophan residues in gramicidin A at positions 15, 13, 11, and 9 with glycine showed that there was no detectable effect at position 15 but increasing heterogeneity of species in the micelles proceeding toward the interior of the micelle at position 9. The replacement of tryptophan at positions 15 and 9 with alanine was found to produce more species heterogeneity than found with glycine substitution at the same positions. An increase in the SDS concentration reduces the number of different species present in micelles. With the Gly-11, Gly-13, and Gly-15 analogs, the increase in SDS concentration results in the formation of a single species; however, for the Gly-9, Ala-9, and Ala-15 analogs, heterogeneity remains.

INTRODUCTION

Small peptides, compared to large proteins embedded into membranes where there are many interactions of side chains with the membrane, must maximize the effects of side-chain interactions with the membrane environment to form stable, solubilized species. With the small peptides, the removal of one residue that strongly interacts with the membrane environment can dramatically change the solubilization and form of the peptide within the membrane. Gramicidin A is a 15-residue hydrophobic peptide that forms transmembrane channels in membranes and model membranes. The amino-acid sequence for gramicidin A is HCO-L ^{1}Val-^{2}Gly-L ^{3}Ala-D ^{4}Leu-L ^{5}Ala-D ^{6}Val-L ^{7}Val-D ^{8}Val-L ^{9}Trp-D ^{10}Leu-L ^{11}Trp-D ^{12}Leu-L ^{13}Trp-D ^{14}Leu-L ^{15}Trp-NHCH$_2$CH$_2$OH (Sarges and Witkop, 1965). When placed in lipid membranes or SDS micelles, gramicidin A forms right-handed, single-stranded $\beta^{6.3}$ helical dimer channels, the monomers being joined at their NH_2 termini (*1–14*). Single amino-acid residue replacement in gramicidin A can produce changes in cation transport and binding (*15–18*). Amino-acid substitution and side-chain orientation are thought to play a significant role in determining the transport properties of the gramicidin channel (*6, 15, 19–29*).

It appears that tryptophan residues are important in modulating both the structure and function of proteins and peptides, such as gramicidin. The hydrophilic and hydrophobic character of the indole side chain of tryptophan permits this residue to partition itself at the aqueous interface of the lipid bilayers (*30–34*). It has also been suggested that the indole NH is directed toward the hydrophilic environment in a number of membrane-bound proteins (*31, 32, 35*). Through the ability to form hydrogen bonds at the aqueous–lipid interface, tryptophan residues at this position provide a necessary structural anchor for that part of the protein or peptide that is near the aqueous environment (*33, 36*). With gramicidin A, it seems that the indole NH moiety of tryptophan may hydrogen bond to the aqueous interface or with the lipid molecules (*37–41*) and that this interaction may stabilize the gramicidin monomer in lipid bilayers (*39*).

Recently, it was shown that the replacement of tryptophan at position 11 in gramicidin A with glycine produces species heterogeneity (i.e., more than a single form of gramicidin) when the peptide is incorporated into sodium dodecylsulfate (SDS) micelles (*42*). It was also observed that the heterogeneity is dependent upon the peptide/SDS ratio. At high SDS concentrations (e.g., peptide/SDS of 3 m*M*/700 m*M*), the heterogeneity almost completely disappears and only one form is observed. For gramicidin A, which has four tryptophan residues, it appears that it is not only the removal of a tryptophan residue but also the type and position of the substitution that is critical in determining the stability, degree of solubilization, and heterogeneity of the species in SDS micelles. We report the results of an investigation of the effects of the replacement of the tryptophan residues at positions 9, 11, 13, and 15 with glycine and the replacement of tryptophan at positions 9 and 15 with alanine on the heterogeneity of gramicidin species in SDS micelles. The dependence of the degree of heterogeneity upon temperature and peptide/SDS concentration ratio was also studied.

1090-7807/97 $25.00

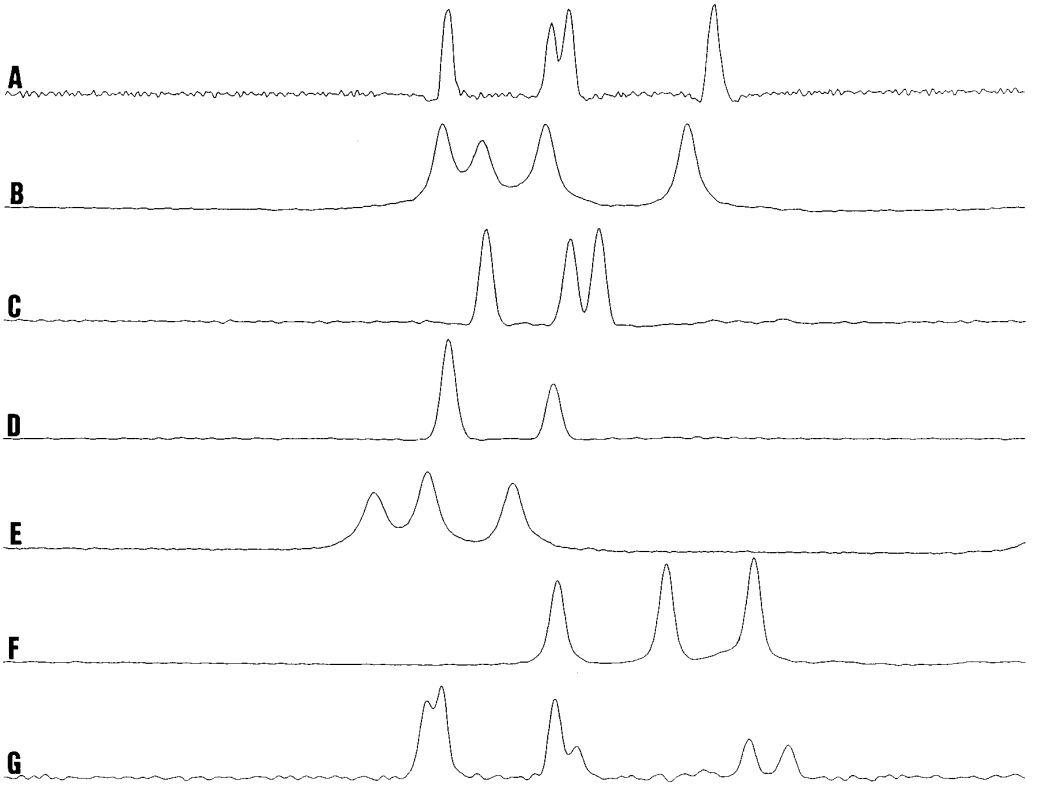

FIG. 1. Indole-NH-proton spectra at 55°C of: (A) gramicidin A, (B) Phe-1 gramicidin A, (C) gramicidin B, (D) gramicidin C, (E) Phe-1 gramicidin C, (F) Gly-15 gramicidin A, and (G) Gly-11 gramicidin A.

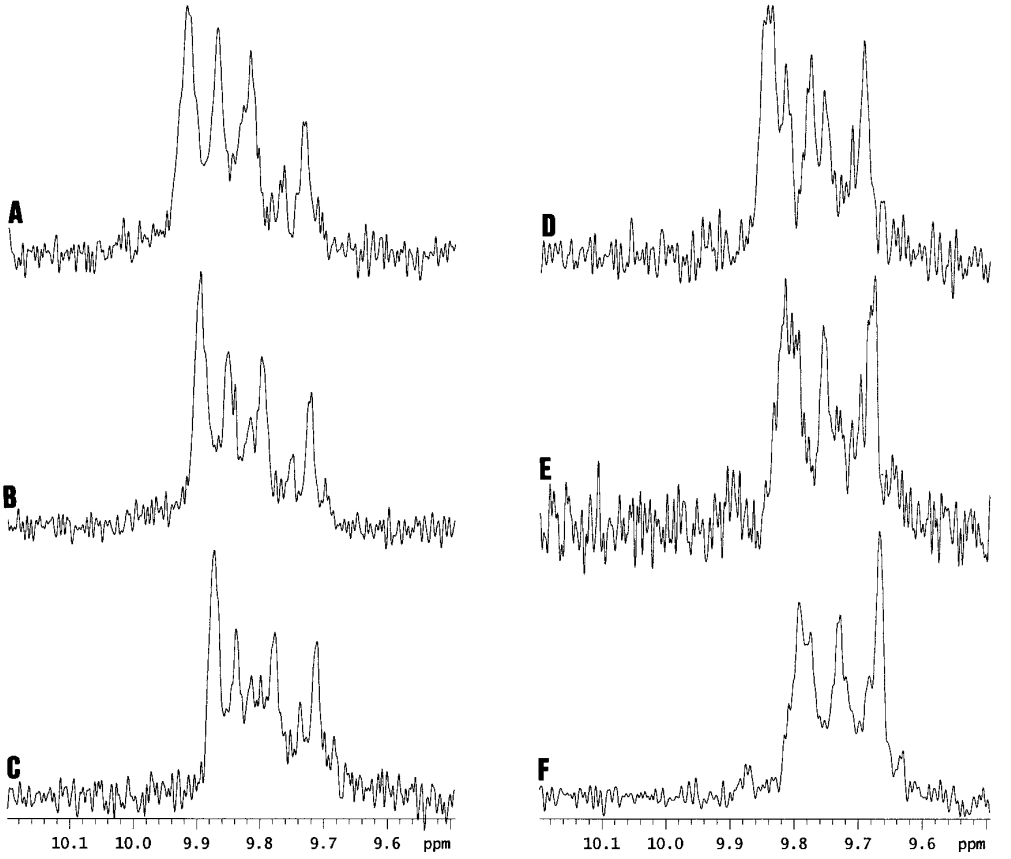

FIG. 2. Indole-NH-proton spectra as a function of temperature of Gly-9 gramicidin A in a sample whose peptide/SDS ratio is 1/135. (A) 30°C, (B) 35°C, (C) 40°C, (D) 45°C, (E) 50°C, and (F) 55°C.

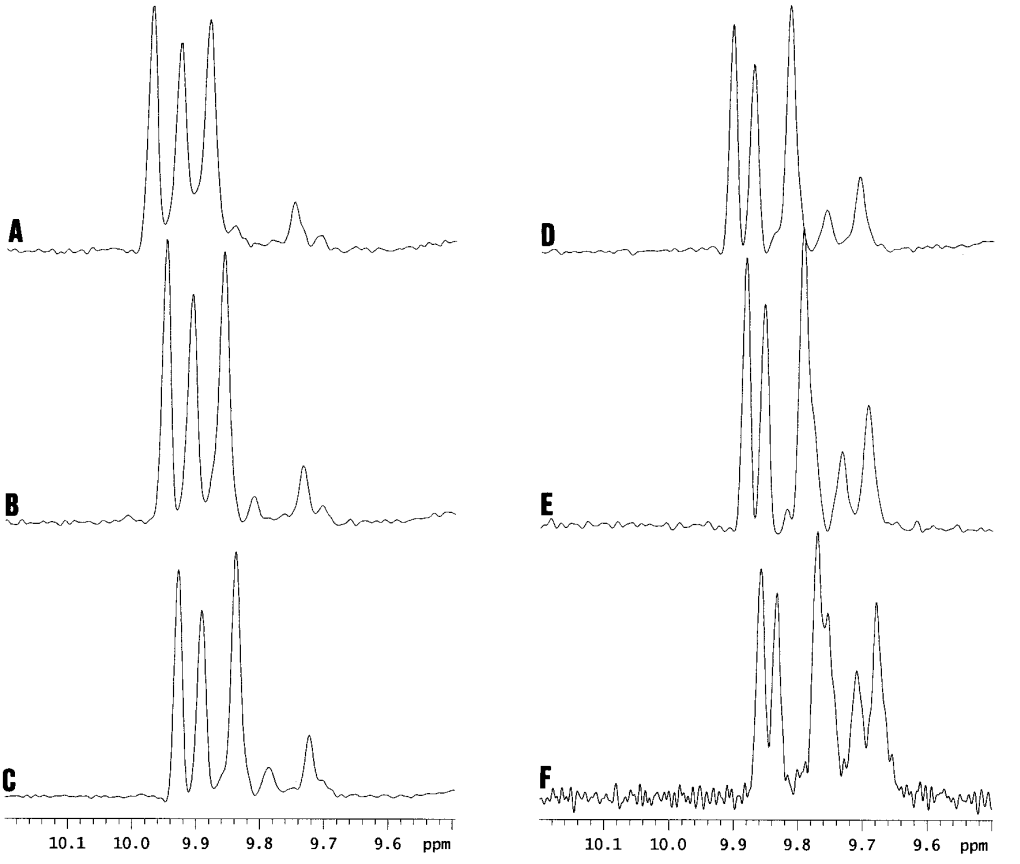

FIG. 3. Indole-NH-proton spectra as a function of temperature of Gly-9 gramicidin A in a sample whose peptide/SDS ratio is 1/307. (A) 30°C, (B) 35°C, (C) 40°C, (D) 45°C, (E) 50°C, and (F) 55°C.

MATERIALS AND METHODS

Methods previously described in the literature (*43–45*) were used to obtain gramicidin A from the commercially available gramicidin D (Sigma Chemical Co., St. Louis, Missouri). The Gly-9, Gly-11, Gly-13, Gly-15, Ala-9, and Ala-15 gramicidin A analogs were synthesized with an Applied Biosystems 431A Peptide Synthesizer (Foster City, California). Sodium dodecyl sulfate$_{-d25}$ (SDS, 98%), trifluoroethanol$_{-d3}$ (TFE, 99%), and deuterium oxide (D_2O, 99,99%) were obtained from Cambridge Isotopes Laboratories (Cambridge, Massachusetts). The SDS was recrystalized from 95% ethanol. A 100 ml pH 6.5 phosphate solution was purchased from PGC Scientifics (Gaithersburg, Maryland). Approximately 25 to 50 m*M* solutions of the gramicidin analogs in TFE were added to 275 m*M* SDS (89% pH 6.5 buffer/11% D_2O v/v). The samples were sonicated in a Cole-Parmer 8851 sonicator for approximately 5 min, then 700 μl of the solution was transferred to a 4 mm NMR tube (Ultra High Precision, 535-PP, Wilmad, Buena, New Jersey). The final concentrations of the samples were approximately 3 m*M* in gramicidin analog, 250 m*M* SDS containing 80% pH 6.5 buffer, 10% TFE, and 10% D_2O v/v/v. For samples of different peptide/SDS ratio, the SDS concentration was adjusted, keeping the peptide concentration at 3 m*M*.

The ^{1}H NMR spectra were obtained with a Varian VXR-500S spectrometer (Palo Alto, California). The first increment of the nuclear Overhauser effect spectroscopy (NOESY) experiment was used to obtain the 1D spectrum

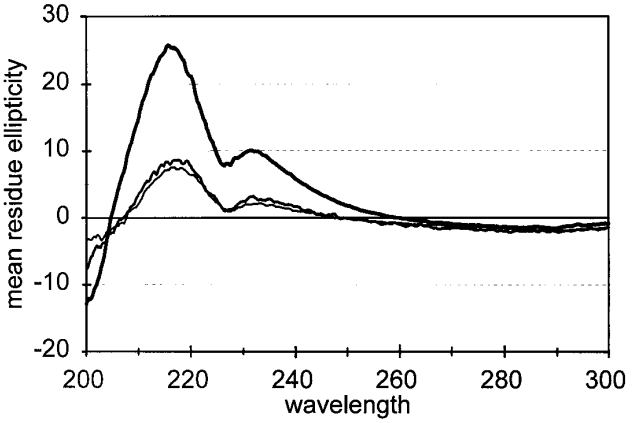

FIG. 4. The CD spectra of gramicidin A (top), Gly-9-gramicidin A in the 1/307 peptide/SDS ratio sample (middle), and Gly-9 gramicidin A in the 1/135 peptide/SDS ratio sample (bottom).

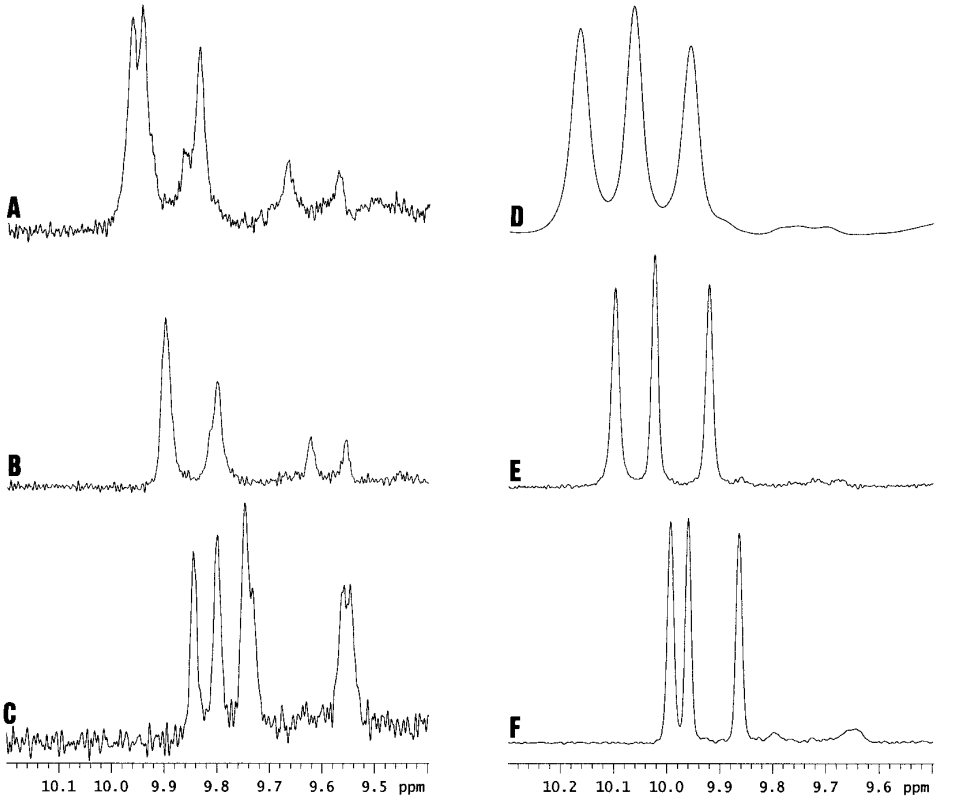

FIG. 5. Indole-NH spectra of Gly-11 gramicidin A in the 1/40 peptide/SDS ratio sample at (A) 30°C, (B) 40°C, and (C) 55°C and in the 1/250 peptide/SDS ratio sample (D) 30°C, (E) 40°C, and (F) 55°C.

of each analog. The NOESY spectra were acquired in 1024 t_1 increments and 8192 t_2 data points. A 7000 Hz spectral width was used in both dimensions in the NOESY experiment. A mixing time of 40 ms was used for all experiments. The samples were allowed to equilibrate for at least 3 h in the probe at each temperature before beginning each experiment. Spectra were obtained twice at each temperature, once as the temperature was increased and then again as the temperature was decreased to the starting temperature. Identical spectra were obtained at each temperature, showing the reversible nature of the change in heterogeneity.

The CD spectra were obtained using a Jasco-700 spectropolarimeter (Jasco, Inc., Maryland). At least eight scans were averaged to obtain the final spectrum.

RESULTS AND DISCUSSION

The indole-NH proton NMR signals of the tryptophan residues in analogs of gramicidin A have been found to be very useful in the study of species heterogeneity because they are well resolved and the chemical shift of these protons is very sensitive to the environment into which they are placed. Figure 1 shows the indole-NH proton spectra of gramicidin A and a number of analogs (i.e., Phe-1 gramicidin A, gramicidin B where tryptophan at position 11 has been replaced by phenylalanine, gramicidin C where tryptophan at position 11 has been replaced by tyrosine, Phe-1 gramicidin C, Gly-15 gramicidin A, and Gly-11 gramicidin A) incorporated into SDS micelles at 55°C and a peptide/SDS ratio of 1/50 (*42*). The three smaller NMR signals in the spectrum of Gly-11 gramicidin A are those of an additional species of the peptide (i.e., heterogeneity) in the micelles. This peak-doubling phenomenon has also been observed with the fd and Pf1 coat proteins from filamentous bacteriophages solubilized in SDS micelles (*46*) and with the M13 coat protein incorporated into deoxycholate micelles (*47*). The peptide/SDS ratio can also affect the degree of heterogeneity. Upon increasing the SDS concentration to a Gly-11/SDS ratio of 1/230, the smaller peaks disappear (*42*). This is consistent with the coat protein results (*46*).

The replacement of the tryptophan residue at position 9 in gramicidin A with glycine produces an analog whose interaction with the micelle environment is much different than that of gramicidin A. For gramicidin A, a peptide/SDS ratio of 1/50 is sufficient to completely solubilize the peptide; however, the Gly-9 analog requires a ratio of 1/135 to solubilize it. Although the Gly-9 analog is incorporated into the SDS micelles at this peptide/SDS ratio, it does not form a single type of species nor does an increase in

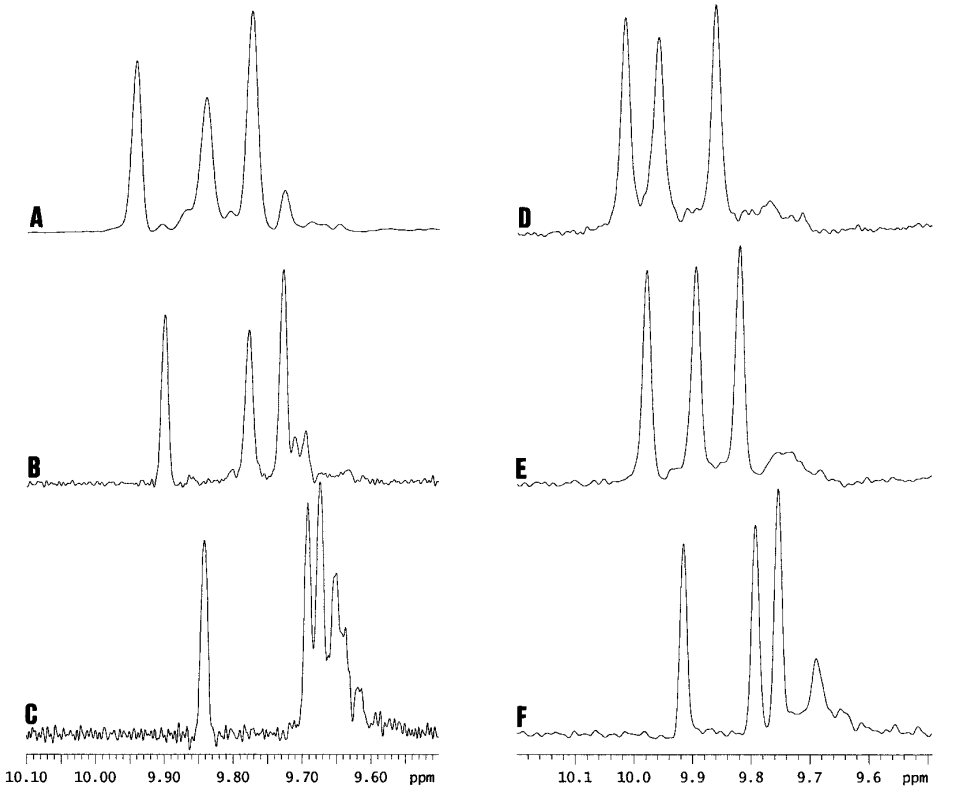

FIG. 6. Indole-NH spectra of Gly-13 gramicidin A in the 1/50 peptide/SDS ratio sample at (A) 30°C, (B) 40°C, and (C) 55°C and in the 1/250 peptide/SDS ratio sample (D) 30°C, (E) 40°C, and (F) 55°C.

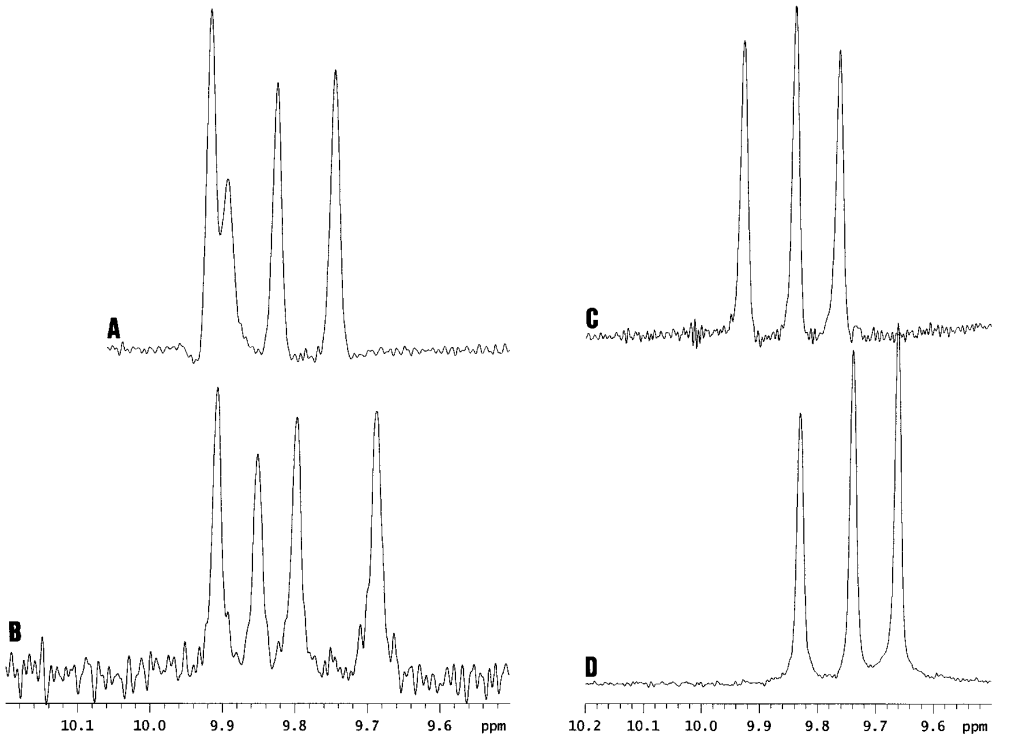

FIG. 7. Indole-NH spectra of gramicidin A in a 1/50 peptide/SDS ratio sample at (A) 30°C and (B) 55°C and Gly-15 gramicidin A in a 1/50 peptide/SDS ratio sample at (C) 30°C, and (D) 55°C.

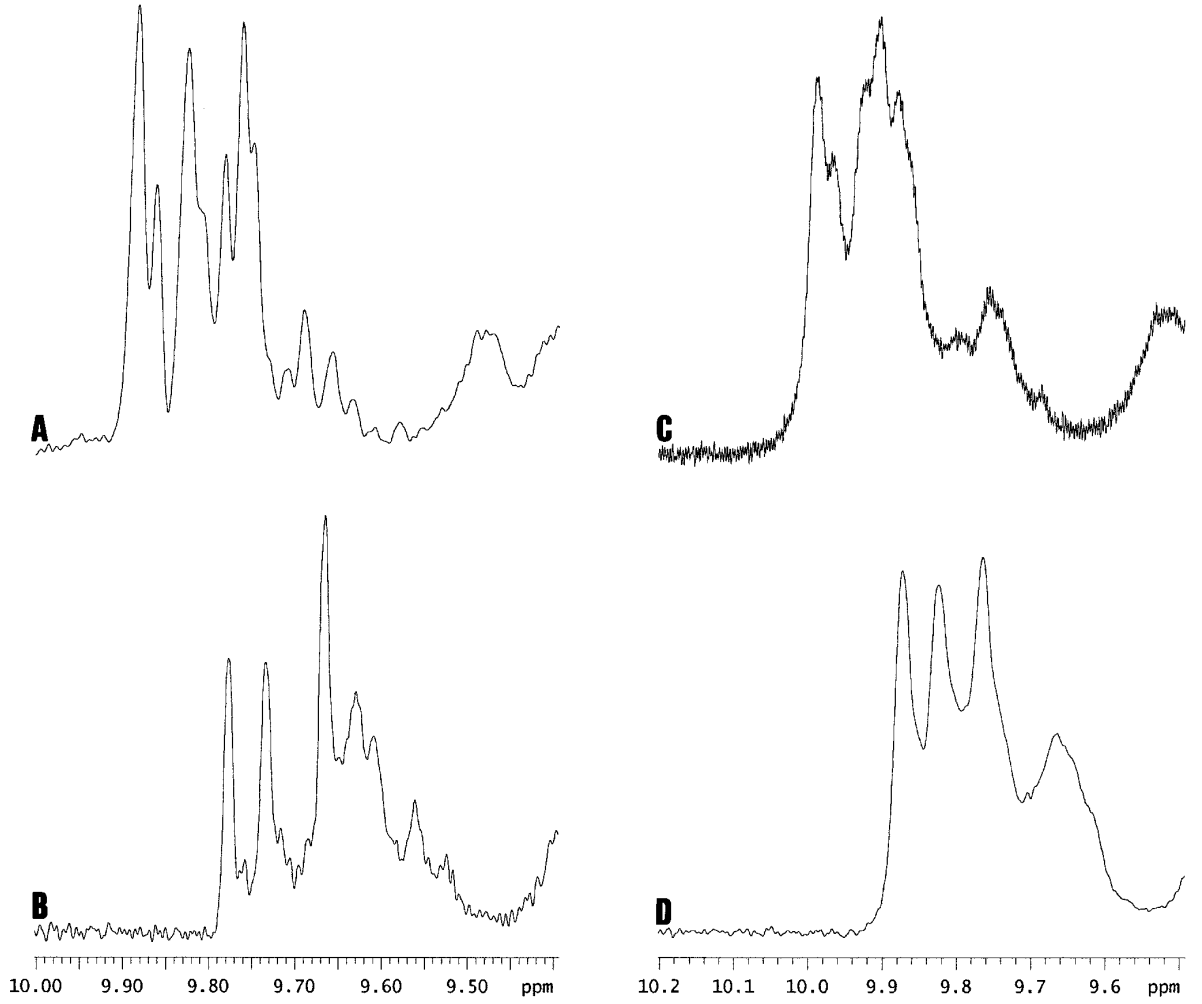

FIG. 8. Indole-NH spectra of Ala-9 gramicidin A in a 1/50 peptide/SDS ratio sample at (A) 30°C and (B) 55°C and in a 1/300 peptide/SDS ratio sample at (C) 30°C and (D) 55°C.

the peptide/SDS ratio of 1/307 (approximately the limit of usable SDS solubility) produce only a single form of the peptide. Figures 2 and 3 show the NH-proton spectra of the Gly-9 analog for the 1/135 and 1/307 peptide ratios. If only one form were present, there should be three indole-NH signals. Obviously, a number of species exist in the 1/135 ratio. Increasing the peptide/SDS ratio to 1/307 decreases this number; however, at least two forms remain. Figures 2 and 3 also show the effect of increasing temperature on the Gly-9 1/135 and 1/307 samples. Increasing the temperature does not appear to force the system into a single species nor do the species appear to interconvert since there is no indication of peak coalescence. The changes observed are the results of the response of hydrogen bonding (i.e., the peaks of the species move to high field in a parallel manner) and relaxation times (i.e., the peaks for the species become narrower) with the increase in temperature.

The CD spectrum of gramicidin A incorporated into SDS micelles at a peptide/SDS ratio of 1/50 and existing as a single-stranded, right-handed $\beta^{6.3}$ helix is shown in Fig. 4. The CD spectra of the Gly-9 analog in the 1/130 and 1/307 peptide/SDS ratio samples are also shown in Fig. 4. The reduced amplitude of the Gly-9 samples is due to one less tryptophan residue in this analog compared to gramicidin A. The similarity of the CD spectra suggests that the Gly-9 forms are β helices. The CD spectra of the Gly-9 samples, however, do not indicate the presence of heterogeneity.

The Gly-11 analog of gramicidin A, where heterogeneity was first observed in the NH-proton spectrum of the indole ring of tryptophan (*42*), exhibits similar, but in a less dramatic manner, characteristics to the Gly-9 analog with respect to the interaction with the SDS micelle environment. The Gly-11 gramicidin A analog, however, is solubilized at a peptide SDS ratio of 1/33 and almost completely converted into a single form at a ratio of about 1/130. The effect of temperature on heterogeneity for Gly-11 is similar to that of Gly-9 as can be seen in Fig. 5.

The analog of gramicidin A that has the tryptophan at

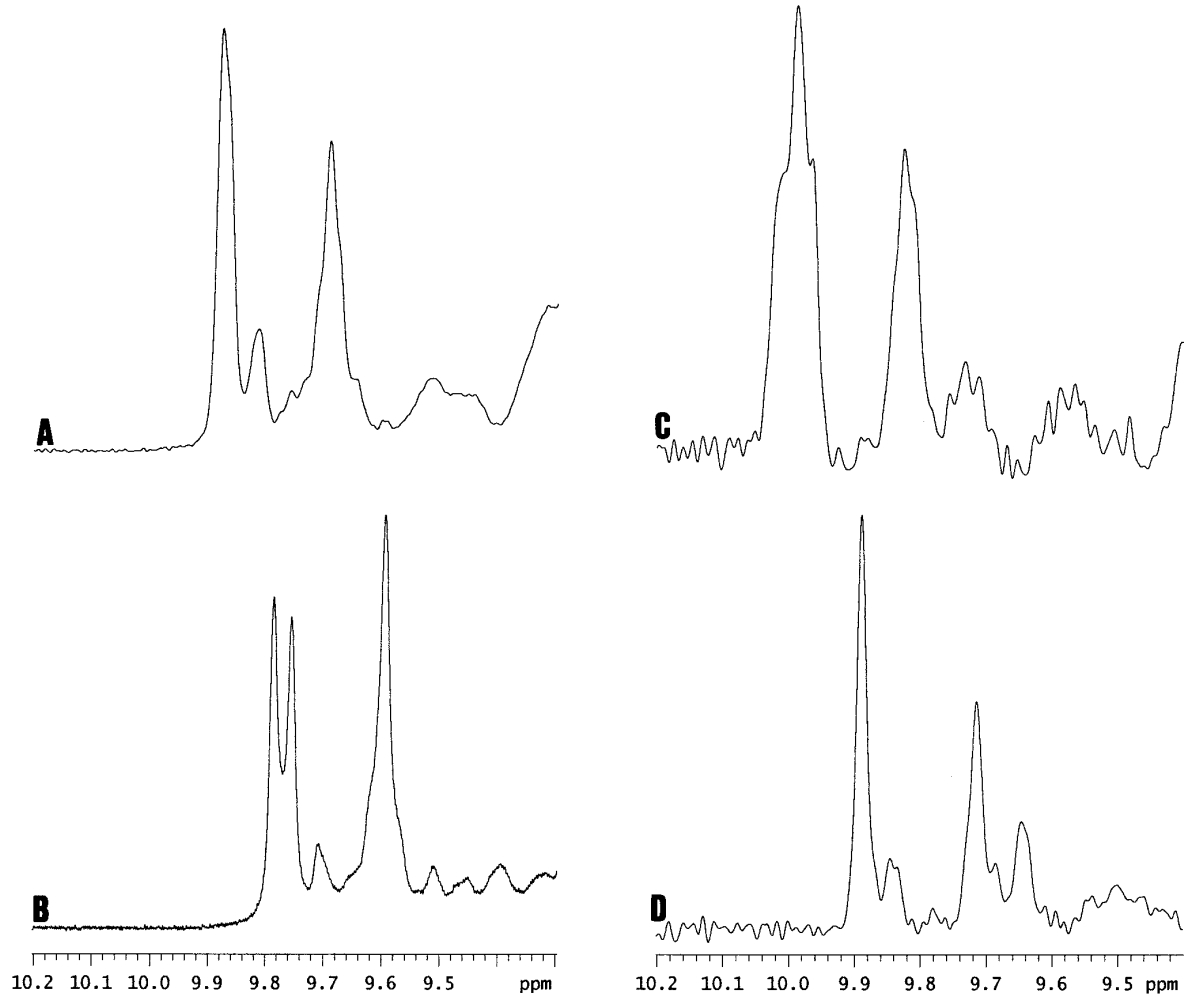

FIG. 9. Indole-NH spectra of ALA-15 gramicidin A in a 1/50 peptide/SDS ration sample at (A) 30°C and (B) 55°C and in a 1/300 peptide/SDS ratio sample at (C) 30°C and (D) 55°C.

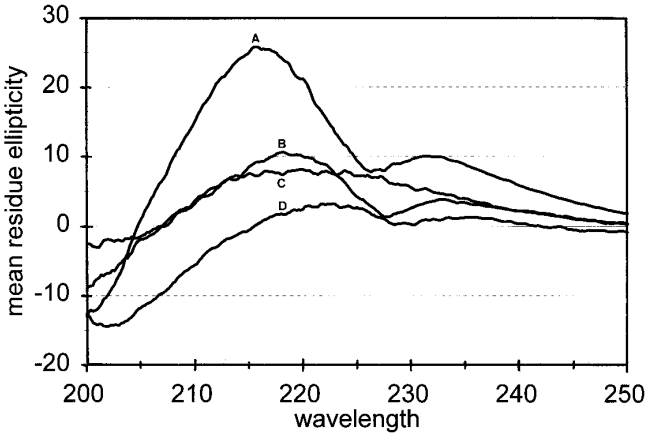

FIG. 10. The CD spectra of gramicidin A in the 1/50 peptide/SDS (A), Ala-9 gramicidin A in the 1/300 peptide/SDS (B), Ala-15 gramicidin A in the 1/300 peptide/SDS (C), and Gly-15 gramicidin A in the 1/50 peptide/SDS (D) samples.

position 13 replaced by glycine exhibits a relatively small degree of heterogeneity that is similar to that of Gly-11. Figure 6 shows the indole-NH-proton spectra of the three tryptophan residues of Gly-13 at peptide/SDS ratios of 1/50 and 1/250 and as a function of temperature. At the higher ratio, the heterogeneity has almost disappeared. The Gly-15 gramicidin A analog did not exhibit any heterogeneity at the 1/50 peptide/SDS ratio, nor at 30 or 55°C. This analog appears to behave like gramicidin A with respect to there being only one species present (Fig. 7). The three-dimensional structure of the Gly-15 analog in SDS micelles, obtained from NOESY distance restraints and molecular modeling, is almost identical to that of gramicidin A.

Replacing tryptophan with alanine increases the level of heterogeneity beyond that observed with glycine replacement. Figures 8 and 9 show the indole NH spectra of the Ala-9 and Ala-15 gramicidin A analogs at the peptide/SDS ratios of 1/50 and 1/300. Even with Ala-15 at the high SDS concentration, heterogeneity persists. The CD spectra (Fig. 10) of the Ala-9 and Ala-15 analogs incorporated into mi-

celles provide no evidence for the presence of heterogeneity when compared to gramicidin A and Gly-15 gramicidin A which form single-species systems that are channels.

Although the emphasis has been upon the use of the indole NH proton signals to indicate the presence of heterogeneity, the phenomenon is not just related to the tryptophan side chain. The backbone NH region of the proton NMR spectrum of the Gly-9, Gly-11, Gly-13, Ala-9, and Ala-15 gramicidin A analogs exhibits many additional peaks compared to the number found in the same spectral region for gramicidin A. Consequently, the heterogeneity is not just related to the side chain of the tryptophans but to the entire molecule.

In summary, the delicate balance of interactive forces that must exist between the side chains of gramicidin and the lipid-like environment of the micelles, critical for the formation of a single species of the gramicidin channel, is a complex function of the type of substitution, position of substitution, and the peptide/SDS ratio. Clearly, a potential problem may arise if one assumes that the solubilization of the peptide in the micelles indicates the existence of a single form of the peptide.

ACKNOWLEDGMENTS

The National Science Foundation is thanked for Grant MCB-9313835. We also thank Dr. D. V. Greathouse for the CD spectrum of gramicidin A.

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