# SOLVENT HISTORY DEPENDENCE OF GRAMICIDIN A CONFORMATIONS IN HYDRATED LIPID BILAYERS

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ABSTRACT Reconstituted lipid bilayers of dimyristoylphosphatidylcholine (DMPC) and gramicidin A' have been prepared by cosolubilizing gramicidin and DMPC in one of three organic solvent systems followed by vacuum drying and hydration. The conformational state of gramicidin as characterized by $^{23}Na$ NMR, circular dichroism, and solid state $^{15}N$ NMR is dependent upon the cosolubilizing solvent system. In particular, two conformational states are described; a state in which $Na^+$ has minimal interactions with the polypeptide, referred to as a nonchannel state, and a state in which $Na^+$ interacts very strongly with the polypeptide, referred to as the channel state. Both of these conformations are intimately associated with the hydrophobic core of the lipid bilayer. Furthermore, both of these states are stable in the bilayer at neutral pH and at a temperature above the bilayer phase transition temperature. These results with gramicidin suggest that the conformation of membrane proteins may be dictated by the conformation before membrane insertion and may be dependent upon the mechanism by which the insertion is accomplished.

## INTRODUCTION

When attempting to make correlations between function, structure, and dynamics for a protein or polypeptide at an atomic level, it is ultimately important to be able to characterize the sample's functional state. The importance of this characterization is amplified for membrane-bound polypeptides and proteins, because it is apparent from the work reported here that these molecules can have more than one energetically stable conformation in a lipid bilayer. Furthermore, because of the possibility of interconverting conformational states the functional characterization needs to be performed with as little perturbation to the samples being used for structural and dynamic studies as possible.

It is well known that the 15–amino acid linear polypeptide, gramicidin A, has different conformations in organic solvents compared with that in a phospholipid environment (Wallace et al., 1981). In this latter environment the functional gramicidin conformation is generally accepted to be an amino terminus–to–amino terminus dimer of helical monomers that forms a monovalent cation-selective channel. The peptide linkages line a pore that is 4 Å in diameter. The structural model for this channel was originally developed by means of conformation analysis in 1971 (Urry). However, because of the difficulty of forming cocrystals of the polypeptide and lipid which diffract to high resolution, confirmation by an atomic resolution structure determination has not been achieved for gramicidin in the presence of lipids and water (Wallace, 1986). This deficiency and the more general problem associated with cocrystallization has prompted structural studies of gramicidin in oriented lipid bilayers by solid state NMR (Cross, 1986*a* and *b*; Nicholson et al., 1987; Fields et al., 1988).

The primary means by which the gramicidin conformation has been monitored in a phospholipid environment is circular dichroism (CD). It is generally agreed upon that the CD spectrum with maxima at 217 and 237 nm and a minimum at 229 nm as well as a negative ellipticity below 205 nm is characteristic of the gramicidin channel state. Within these general constraints there is considerable variation in the published spectra depending on how the samples are prepared (Urry et al., 1979*a* and *b*; Masotti et al., 1980; Wallace et al., 1981; Wallace, 1984). Typically, samples that have been heated to form the channel state have a very shallow minimum at 229 nm with a positive ellipticity, whereas samples that have been prepared by cosolubilization in chloroform/methanol have a more pronounced minimum with a negative ellipticity. CD is limited to characterizations of samples having low light scattering properties such as micelles or small vesicles. Consequently, the CD spectrum is obtained after sonication and depending on either the heat treatment conditions or the solvent system used for cosolubilization, the CD result will vary substantially.

A second approach for characterizing the gramicidin conformation is $^{23}Na$ NMR which has been used for monitoring the conformation in micelles and vesicles (e.g., Urry et al., 1979*b*; Masotti et al., 1980; Urry et al., 1982; Urry et al., 1985). When gramicidin is in the channel conformation the $^{23}Na$ resonance is broadened, shifted, and its $T_1$ relaxation time substantially shortened. These spectroscopic changes are reversed when $AgNO_3$ or some other salt that blocks the transport of monovalent cations

by gramicidin is added to the samples (Urry et al., 1979*b*). Whereas this spectroscopic technique has previously only been applied to low light scattering systems, it is not restricted to such use and therefore it could be considered as an approach for characterizing the gramicidin conformation in extensive lipid bilayer samples. This would mean that samples used for solid state $^{15}N$ NMR spectroscopy could be characterized without having to vesicularize the samples by sonication or the addition of detergent which may alter the conformational state of the gramicidin.

Previous reports have suggested that to obtain the CD and $^{23}Na$ NMR results indicative of the channel state it has been necessary to incubate the phospholipid/gramicidin complex at temperatures around 70°C for 8–20 h (Urry et al., 1979*a* and *b*; Spisni et al., 1979; Masotti et al., 1980; Urry et al., 1985). These studies utilized samples prepared by adding gramicidin either as a dry powder or in organic solvents to preformed micelles or vesicles. Other laboratories have prepared samples by cosolubilizing gramicidin and phospholipid in an organic solvent system, followed by drying and hydrating the mixture. Such preparations do not always require extensive incubation at elevated temperatures to achieve the characteristic spectral features of the gramicidin channel state. In the effort reported here three different cosolubilizing solvents, chloroform/methanol, benzene/methanol, and trifluoroethanol (TFE), have been used. In the past chloroform/methanol has been most extensively used for this purpose (Weinstein et al., 1980; Wallace et al., 1981; Wallace, 1984; Shungu et al., 1986). Benzene/methanol has been used by B. A. Cornell and co-workers (Smith and Cornell, 1986). Trifluoroethanol was suggested by Urry and Killian (Tournois et al., 1987) because of the monomeric nature of gramicidin in TFE solution (Urry et al., 1972; Glickson et al., 1972). In a recent $^{23}Na$ NMR study large unilamellar vesicles containing gramicidin were prepared by the addition of a TFE solution of gramicidin to preformed vesicles (Buster et al., 1988).

Both CD and $^{23}Na$ NMR are used in this study for characterizing the gramicidin/lipid samples, as well as three other approaches. $^{31}P$ NMR is used to document that the lipid phase is that of a bilayer. Differential scanning calorimetry (DSC) is used to document that the polypeptide is incorporated into the hydrophobic domain of the phospholipids. Solid state $^{15}N$ NMR spectra of uniformly $^{15}N$ labeled gramicidin in lipid bilayers aligned with the bilayer normal parallel with the magnetic field of the spectrometer are used to provide a fingerprint for the backbone conformation. The chemical shift of each $^{15}N$ site is sensitive to the dynamics and orientation of the site with respect to the bilayer normal. Unlike the interpretation of an isotropic chemical shift which is frequently influenced by a great many poorly defined factors the chemical shift frequency of an oriented sample is very well defined. In such samples the frequency is dominated by the orientation of the site with respect to the magnetic field and the molecular motion which averages the chemical shift interaction. Consequently, any change in the conformational or dynamic state of gramicidin which affects the backbone will result in changes to the solid state $^{15}N$ spectrum. One of the achievements of this study is to document the solid state $^{15}N$ spectrum of gramicidin in the channel state. However, the primary achievement is to show that different conformations of gramicidin can exist in the hydrophobic interstices of a phospholipid bilayer, and that the most stable conformation achieved by a variety of preparative routes is the channel state. The fact that other states can exist demonstrates the need for characterization techniques which require a minimum of sample manipulation.

## MATERIALS AND METHODS

Uniformly $^{15}N$ labeled gramicidin A′ was produced biosynthetically by *Bacillus brevis* (ATCC 10068) as previously described (Nicholson et al., 1987). A modification of the purification scheme was implemented to forgo cell lysis with trichloroacetic acid and to prevent acid oxidation of the trytophan sidechains. Instead, the cells were lysed by the addition of 125 ml of 4 M KOH per liter of bacterial growth.

Natural abundance gramicidin A′ was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. DMPC was purchased from either Sigma Chemical Co. or Avanti Polar Lipids (Birmingham, AL) and used without further purification. All gramicidin/DMPC samples were prepared in a 1:8 molar ratio by cosolubilizing the polypeptide and lipid in one of three solvent systems followed by drying under vacuum and hydration. The three solvent systems were 97% chloroform/3% methanol, 97% benzene/3% methanol, and 100% trifluoroethanol. Excess drying time was taken to ensure that drying was complete. Hydration of the lipid bilayers was accomplished either with deionized water (for the $^{15}N$ NMR and CD samples) or with a 30-mM NaCl solution (for the $^{23}Na$ and $^{31}P$ NMR and the DSC experiments). Hydration to 60% water (vol/wt) was achieved without resorting to agitation by incubating the samples above the gel to liquid crystalline phase transition for 48 h.

Oriented samples were prepared by drying the organic solutions containing the gramicidin/DMPC mixture on glass coverslips (Corning Glass Works, Corning, NY) having dimensions of 5.6 by 12 mm. 25 of the coverslips were stacked in a 12-mm section of square glass tubing and enough water was added to achieve 60% hydration before the tubing was sealed with epoxy. This approach is a minor modification of that described in Nicholson et al. (1987).

For the CD experiments 2.5 ml of deionized water was added per milligram of gramicidin in hydrated bilayers. These solutions were sonicated for 30 min with a Micro Ultrasonic Cell Disruptor (Kontes Co., Vineland, NJ) using a power level of 8 W. The gramicidin/lipid solution temperature was maintained at 38°C and monitored frequently by means of a thermocouple. After sonication the solution was centrifuged to sediment the titanium particles and large light scattering aggregates (trace amount). After recording the CD spectra, samples were incubated overnight at 65°C to try to induce formation of the channel state. In other experiments unsonicated samples were also incubated using the same protocol. CD spectra were recorded on a J-500C spectropolarimeter (Jasco Inc., Easton, MD). DSC of the bilayer preparations was performed on a scanning calorimeter (Perkin Elmer Corp., Norwalk, CT).

$^{31}P$ and $^{23}Na$ NMR spectra were recorded on a homebuilt spectrometer utilizing a wide bore 3.5 Tesla magnet. The $^{31}P$ 90° pulse width was 15 $\mu$s at a frequency is 61 MHz. $^{1}H$ decoupling of $^{31}P$ was achieved with 5 W at 151 MHz and the recycle delay was 5 s. $^{23}Na$ NMR spectra were obtained at 40 MHz using a quadrupolar spin echo sequence (Davis et al., 1976; Solomon, 1958). A $\tau$ value of between 20 and 60 $\mu$s and a recycle delay of

70 ms were used for all of the $^{23}Na$ spectra. A homebuilt probe without glass was required for the $^{23}Na$ spectra to avoid severe baseline distortions.[15]N NMR spectra were recorded on a modified IBM/Bruker WP200 SY spectrometer with a solids package (modifications given in Nicholson et al., 1987). A homebuilt static (i.e., non-magic angle spinning) probe with variable temperature control was constructed for observing both oriented and unoriented samples. Typical experimental conditions utilized a sweep width of 62.5 kHz, preacquisition delay of 16 $\mu$s, and a recycle delay of 7 s. Spectra were obtained by cross-polarization with fields generated by 4.6 $\mu$s 90° pulse lengths, a mixing period of 1 ms and an increased $^1H$ decoupling field during acquisition of 2.0 mTesla. $^{15}N$ chemical shifts are given relative to the resonance of a saturated solution of $^{15}NH_4NO_3$.

## RESULTS

An outline of the sample treatment and interpretation of the various spectroscopic and calorimetric results is presented in Fig. 1. Hydration of the gramicidin/DMPC samples was performed with a minimum of agitation (i.e., no vortexing or swirling of the flask) so that the samples would contain extensive lipid bilayers and not vesicles or relatively small multilamellar liposomes. These samples were then characterized by $^{31}P$ NMR as to the lipid phase, by DSC as to the incorporation of the gramicidin into the lipid bilayer, and by $^{23}Na$ NMR to document the presence or absence of the gramicidin channel state. A portion of these samples was incubated overnight at 65°C to see if the samples cosolubilized in benzene/methanol and chloroform/methanol could be converted to the channel state. Other bilayer samples were prepared and sonicated to minimize light scattering for CD studies that were performed before and after heat treatment to determine the presence or absence of the channel state.

The $^{31}P$ chemical shift powder patterns for three bilayer samples prepared from different solvent systems are presented in Fig. 2. Each sample clearly shows that the bilayer phase is dominant and that there are no hexagonal phase lipids. The observed anisotropy of 34–36 ppm is independent of the cosolubilizing solvent. These values are dependent upon achieving a uniform level of hydration and this process requires considerably more hydration time when the solvent is chloroform or benzene than when it is trifluoroethanol. This value is consistent with that obtained by Rajan et al. (1981) and Nicholson et al. (1987).

The DSC thermograms show phase transition temperatures of 28°C for the lipid and 26°C for the three gramicidin/DMPC bilayer preparations from different solvents as seen in Fig. 3. The width of the transition is markedly increased when gramicidin is present, in agreement with previous studies, and the enthalpy of the transition is greatly reduced (Chapman et al., 1977; Nicholson et al., 1987). Because the phase transition represents a change in the physical state of the hydrophobic interstices of the bilayer, these dramatic changes in the thermograms are a clear indication that the polypeptide in each of the gramicidin samples is in intimate contact with the fatty acyl chains of the lipids.

The $^{23}Na$ NMR linewidth results obtained from the bilayer preparations (Fig. 4) indicate that there is a very dramatic difference between the three solvent systems used for cosolubilization. The samples are very viscous, being ~60% water (vol/wt), and consequently the $^{23}Na$ resonance even from a sample of DMPC bilayers in the absence of gramicidin is relatively broad (~2 ppm). However, when TFE is used as the cosolubilizing solvent for samples of gramicidin and lipid, then the resulting $^{23}Na$ resonance is further broadened (30 ppm), whereas the use of benzene/methanol and chloroform/methanol result in a

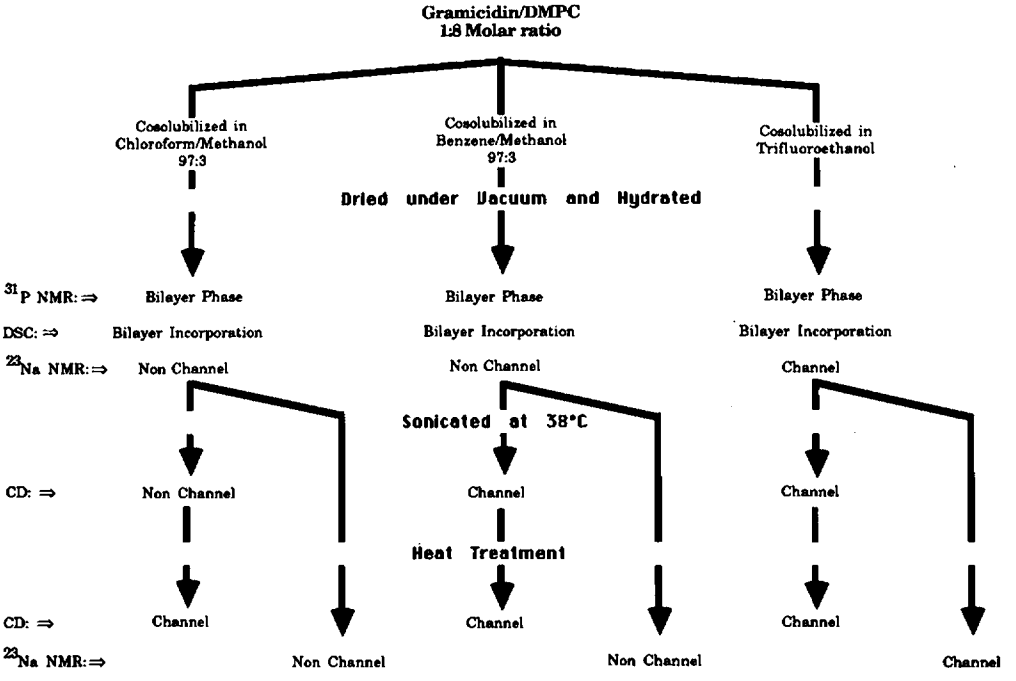


FIGURE 1 Flow chart summarizing the gramicidin sample manipulation, the interpretation of the differential scanning calorimetry, and the $^{23}Na$ and $^{31}P$ nuclear magnetic resonance spectra. Details of the sample manipulations are given in Methods and Materials, while the results are explained in Results and Discussion.

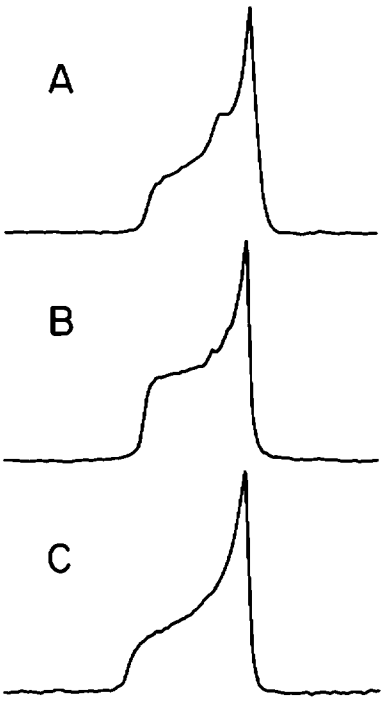


FIGURE 2 $^{31}P$ NMR powder pattern spectra of hydrated DMPC bilayers containing gramicidin above the phase transition temperature centered at 26°C. (*A*) Sample cosolubilized in 97% chloroform/3% methanol. (*B*) Sample cosolubilized in 97% benzene/3% methanol. (*C*) Sample cosolubilized in 100% trifluoroethanol. In all three spectra the width of the motionally averaged powder pattern is between 34 and 36 ppm.

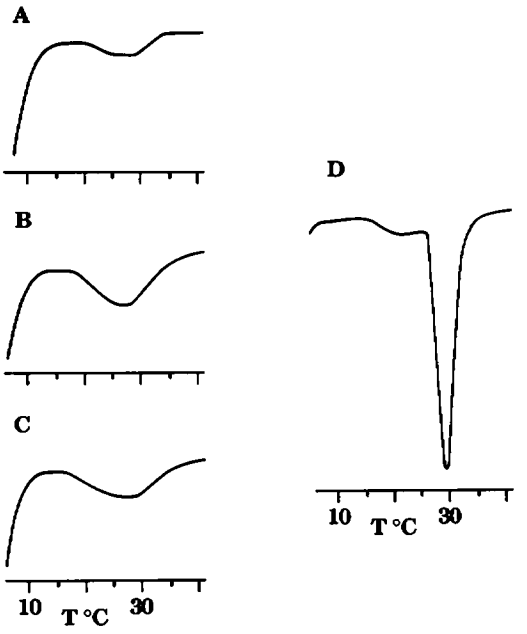


FIGURE 3 Differential scanning calorimetry thermograms of hydrated gramicidin containing DMPC bilayers (*A*, *B*, and *C*) and hydrated DMPC bilayers alone (*D*). A heating rate of 10°C/min was used for each trace except *D* which was run at 5°C/min. *A*, *B*, and *C* represent three cosolubilizing solvents; 97% chloroform/3% methanol, 97% benzene/3% methanol, and 100% trifluoroethanol, respectively. The sensitivity of the calorimeter was increased by a factor of 5 for the gramicidin containing samples. Each sample contained ~5 mg of DMPC.

linewidth (4–5 ppm) that is not much greater than that observed in the absence of gramicidin. The linewidth is dependent upon several factors including temperature, the relative concentration of gramicidin to $Na^+$, and the correlation time of the gramicidin/lipid aggregate. These experiments were performed at room temperature with a relative molar concentration of gramicidin to $Na^+$ of 2.5 to 1, respectively. The vastly increased linewidths observed here for TFE cosolubilized gramicidin compared with those reported in the literature in vesicle or micellar solutions (e.g., Urry et al., 1979*b*; Masotti et al., 1980) are a result of the very long correlation times associated with the extensive lipid bilayers. But, only when $Na^+$ interacts with the gramicidin in these lipid bilayers does the linewidth increase so dramatically. Consequently, when chloroform/methanol or benzene/methanol is used as a cosolubilizing solvent system $Na^+$ does not interact substantially with the gramicidin channel.

Extensive incubation of these samples at 65°C for ~20 h changes the $^{23}Na$ linewidths very little (Fig. 4, *D–F*) except for a further broadening of the resonance from the TFE-cosolubilized sample (to 56 ppm). It has been shown previously that these conditions, along with the addition of sonication, have been sufficient to generate the channel conformation (e.g., Shungu et al., 1986; Spisni et al., 1979). However, it is apparent that incubation of the

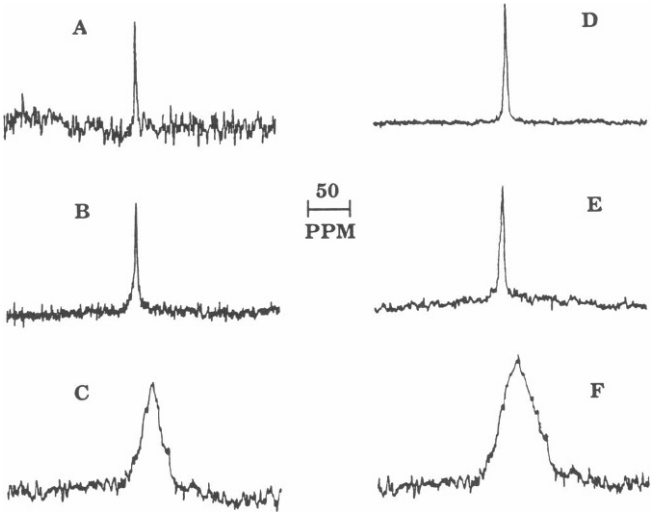


FIGURE 4 $^{23}Na$ NMR spectra of hydrated gramicidin containing DMPC bilayers recorded at 30°C. The $Na^+$ concentration was maintained relative to gramicidin at a molar ratio of 1:2.5 for all samples. (*A* and *D*) Cosolubilized in 97% chloroform/3% methanol. (*B* and *E*) Cosolubilized in 97% benzene/3% methanol. (*C* and *F*) Cosolubilized in 100% trifluoroethanol. *A*, *B*, and *C* were recorded before heat treatment. *D*, *E*, and *F* were recorded after incubation overnight at 65°C. All spectra are displayed with the same frequency scale.

benzene/methanol and chloroform/methanol cosolubilized samples of gramicidin and DMPC does not result in preparations where $Na^+$ interacts substantially with gramicidin.

When the bilayer preparations are diluted and sonicated, CD spectra can be obtained. The results for benzene/methanol (Fig. 5 *B*) and TFE (Fig. 5 *C*) show spectra that are typical of the channel conformation, whereas the spectrum of the chloroform/methanol (Fig. 5 *A*) is very different. However, even this latter preparation can be converted to the spectrum typical of the channel conformation when the sample has been incubated at 65°C overnight (Fig. 5 *D*). The other samples are not significantly affected by the incubation period as seen by the spectra in Fig. 5, *E* and *F*.

The solid state $^{15}N$ NMR spectra of oriented lipid bilayers containing uniformly $^{15}N$ labeled gramicidin are shown in Fig. 6. There are 20 nitrogens in a gramicidin monomer, 16 of which are in the amide peptide linkages of the backbone and four of which are in the indole rings of the tryptophan sidechains. These spectra present a series of sharp resonances from highly oriented regions of the sample (mosaic spread = ±1–3°). Unoriented regions of the sample preparation, in which all possible orientations of the $^{15}N$ sites exist, generate powder pattern intensity over a wide frequency range. Consequently, associated with each sharp resonance is a very broad resonance. For instance, a site which gives rise to a sharp resonance at 198 ppm also gives rise to a broad resonance extending from 198 to 55 ppm, and a resonance at 146 ppm has a broad resonance extending from 146 to 80 ppm associated with it. The amount of powder pattern varies somewhat from sample to sample due to the ratio of unoriented to oriented material in the samples.

Complete resonance assignments are not available at this time. However, a partial distinction between amide and indole sites is possible. The static powder pattern for amide sites extends from ~205 to 40 ppm (Cross and Opella, 1985), whereas the indole powder pattern extends from 150 to 40 ppm (Cross and Opella, 1983). Consequently, the resonances at 198 and 180 ppm, which account for ~6 of the 20 nitrogen sites, must result from the polypeptide backbone. In fact, one of the resonances at 198 has recently been identified using a single site $^{15}N$ labeled gramicidin as the backbone amide of $Ala_3$ (Fields et al., 1988). The major resonances at 198, 180, and 146

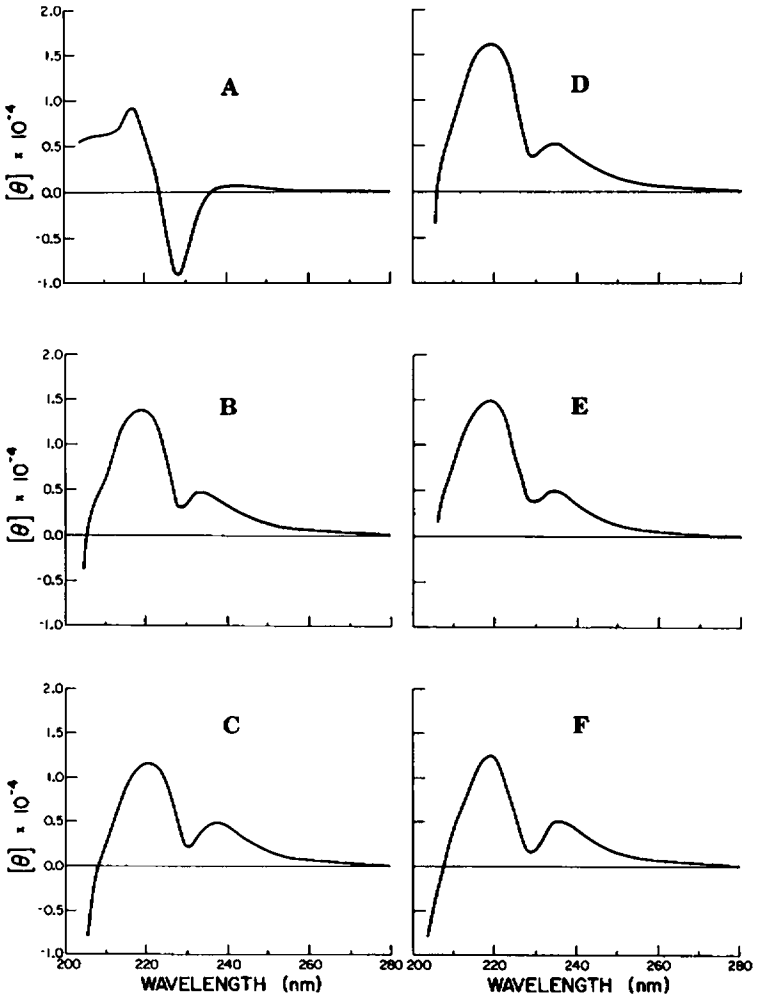


FIGURE 5 Circular dichroism of sonicated gramicidin/DMPC bilayers recorded at 30°C. (*A* and *D*) Cosolubilized in 97% chloroform/3% methanol. (*B* and *E*) Cosolubilized in 97% benzene/3% methanol. (*C* and *F*) Cosolubilized in 100% trifluoroethanol. *A*, *B*, and *C* were recorded before heat treatment. *D*, *E*, and *F* were recorded after incubation overnight at 65°C. In all but part *A* the CD spectra are typical of what is considered to be the channel conformation.

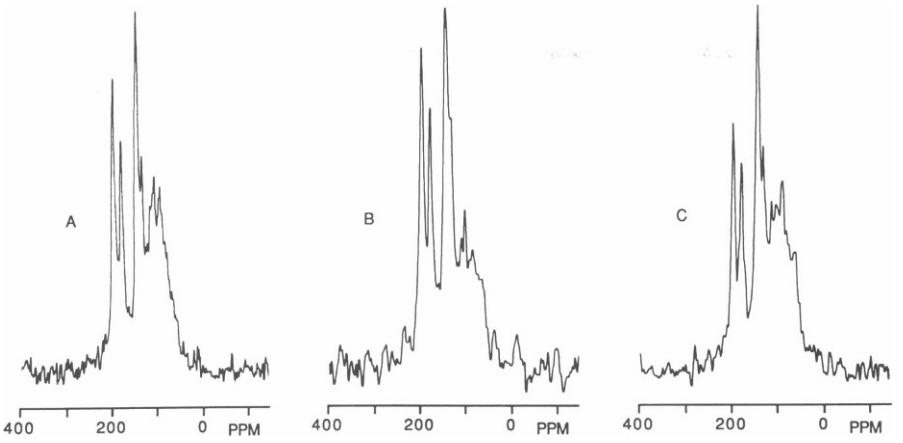


FIGURE 6 $^{15}N$ solid state NMR spectra of uniformly $^{15}N$ labeled gramicidin in DMPC bilayers aligned with the bilayer normal parallel with the magnetic field of the NMR spectrometer. (*A*) Sample cosolubilized in 97% chloroform/3% methanol. (*B*) Sample cosolubilized in 97% benzene/3% methanol. (*C*) Sample cosolubilized in 100% trifluoroethanol. The variable amount of broad intensity that is most prominent on the upfield side of each spectrum results from powder pattern intensity originating from unoriented regions of the sample.

ppm have a constant chemical shift regardless of the cosolubilizing solvent. The relative integrated intensities of these three peaks also remain constant. Furthermore, a number of spectral details such as the shoulder at 131 ppm and peaks at 113 and 104 ppm are present in all three spectra. The "peaks" at ~90 and 60 ppm are the result of the powder pattern resonance, which has a much greater intensity on the high field side of these spectra.

Whereas it is not possible to quantitatively interpret the chemical shifts, because the orientation of the chemical shift tensor is poorly defined relative to a molecular frame of reference, these chemical shifts are very sensitive to structural and dynamic changes in the sample. For instance, a change in the position of the 146 ppm peak by 5 ppm could indicate a change in the orientation of the N-H bond by as little as 2°. The extreme sensitivity of the resonance frequency to the orientation of the nuclear site provides a sensitive fingerprint for the conformation and dynamics of the polypeptide backbone. The backbone structure of a polypeptide can be considered as a series of linked planes whose relative orientation is given by the $\phi$ and $\psi$ torsion angles. Therefore, any changes in these torsion angles will result in changes in the orientation of the $^{15}N$ site within the peptide linkage plane and a consequential change in the resonance frequency.

## DISCUSSION

From the results it is clear that the conformation of gramicidin is dependent upon the solvent used for cosolubilizing gramicidin and lipid. Despite great care in being sure that the organic solvent has been completely removed from the sample, the conformation in the final hydrated bilayers is solvent history dependent. Previous efforts have shown that even a considerable percentage of organic solvent (e.g., 40% methanol) is not enough to change the CD spectral results for gramicidin in hydrated bilayers (Wallace et al., 1981). Consequently, the conformation of gramicidin in the hydrated lipid bilayers is dependent upon the conformation of gramicidin in the dry sample after the cosolubilization and drying, but before hydration. It is known from CD and NMR experiments that the conformation of gramicidin in organic solvents is highly dependent upon the specific solvent used. For instance, Veatch and collaborators (Veatch et al., 1974; Fossel et al., 1974) have demonstrated that four different conformations of a gramicidin dimer could be isolated from methanol solution of gramicidin by thin layer chromatography. An x-ray crystal structure of gramicidin complexed with $Cs^+$ has been determined from crystals derived from a methanol solution (Wallace, 1986). To date this is the only x-ray crystal structure of gramicidin. However, it has been shown that in dimethylsulfoxide (DMSO) solution gramicidin is a monomer and has a $\beta$ helical structure as determined by two-dimensional solution NMR studies (Hawkes et al., 1987). Previous work has suggested that the structure of gramicidin in DMSO and TFE is the same (Glickson et al., 1972; Urry et al., 1972). No other detailed structural analyses have been performed on gramicidin, but it is clear from a variety of CD and solution NMR spectroscopic studies that the conformation of gramicidin in organic solution is highly variable.

The history dependence of the gramicidin conformation has some very interesting implications for other membrane-soluble polypeptides and proteins. The conformation present in the hydrated bilayers is either the organic solvent conformation or an intermediate state between the organic solvent conformation and the channel state. The channel state appears to be the end product conformation in all of the sample preparations studied here. Once formed, the channel state has never been converted to the nonchannel state without redissolving the sample in an organic solvent that favors nonchannel conformations. One of the implications from this effort is that conformational states other than the state associated with a global energy minimum can be trapped in a lipid bilayer. Consequently, in very general terms the conformation of a membrane-bound polypeptide or protein may be dependent upon the mechanism by which it is inserted into the membrane or on the conformation before membrane insertion. It cannot be assumed that once the polypeptide or protein has been

inserted into the membrane that its structure will rearrange to achieve the global energy minimum. This ability of the lipid bilayer to trap high energy conformations may largely be due to an increased energy associated with hydrogen bonds in an apolar environment. Further implications of this result are beyond the scope of this paper, however, it is interesting to speculate that peptide antibiotics of membrane proteins might be stored in membranes in an inactive conformation by the cells producing them.

The CD spectrum of gramicidin/DMPC prepared out of chloroform/methanol has been previously published (Wallace et al., 1981) and is significantly different from that shown in Fig. 5 *A*. While this published spectrum has a positive molar ellipticity at 237.5 nm of $3.8 \times 10^3$, indicative of the channel state, it also has a negative molar ellipticity of $-3 \times 10^3$ at 229 nm which is not indicative of the channel state. Despite this negative ellipticity, Wallace et al. concluded that this is a spectrum of the channel conformation. The spectrum in Fig. 5 *A* shows evidence for a trace amount of the channel conformation as indicated by the small but significant positive ellipticity at 240 nm, but this sample is clearly dominated by a nonchannel gramicidin conformation. Furthermore, the rest of the spectra in Fig. 5 all show a positive ellipticity at 229 nm, similar to many published CD spectra that have been interpreted as indicating the channel state (e.g., Wallace, 1984; Masotti et al., 1980). Consequently, the spectrum in Wallace et al. (1981) is most likely the result of a mixture of predominantly channel and some nonchannel conformations. Several possibilities exist for the difference in the CD spectra obtained in the two laboratories. The most likely reason is that the molar ratio of gramicidin to DMPC is 1:8 for this study and 1:30 in Wallace et al. (1981). If this is in fact the reason for the discrepancy then it implies that the nonchannel conformation is stabilized by a higher ratio of gramicidin to lipid.

While incubation at elevated temperature was very successful at converting the CD spectrum obtained from the sonicated chloroform/methanol sample to a spectrum indicative of the channel conformation, it was not successful at converting the $^{23}$Na NMR spectrum of the chloroform/methanol and benzene/methanol bilayer samples to that indicative of the channel state. With the sample conditions described here, heat treatment is an effective means for generating the channel conformation when gramicidin is in a dilute solution of vesicles, but not when the samples are a concentrated preparation of extensive bilayers. The recent work of Shungu et al. (1986) showed that heat treatment was effective at converting a relatively concentrated solution (80% water [vol/wt]) of DMPC vesicles containing gramicidin at a molar ratio of 1:20 to the channel conformation. Consequently, the effectiveness of heat treatment does not appear to be dependent on the extent of sample hydration, but rather on the physical state of the lipids (i.e., extensive bilayers vs. vesicles) or on the molar ratio of gramicidin to lipid. This later possibility is consistent with the findings above that a high relative concentration of gramicidin to lipid may stabilize the nonchannel conformation.

When TFE is used as the cosolubilizing solvent all of the spectral parameters observed appear to indicate the channel conformation. For chloroform/methanol the $^{23}$Na linewidth results for the bilayer samples show that gramicidin is in a nonchannel conformation and when the sample is diluted and sonicated it remains in a nonchannel conformation. Only after it has been diluted and sonicated can it be converted to the channel conformation by heat treatment. Again, for benzene/methanol the $^{23}$Na linewidth results for the bilayer samples indicate that the gramicidin is in a nonchannel conformation, but when the sample is diluted and sonicated it is converted to the channel conformation as characterized by the CD spectrum. Even though the temperature of the samples was carefully monitored using a thermocouple and controlled by a circulating water bath, the energy input to the sample by sonication was enough to alter the gramicidin conformation. Consequently, any protocol for characterization of the gramicidin channel state in extensive bilayer samples should avoid sonication.

There are multiple advantages in using $^{23}$Na NMR to characterize the functional state of the gramicidin. The first and most significant advantage is that sonication can be avoided. Secondly, there is not a well-understood correlation between structure and function, and therefore observations of the conformational state are a relatively poor means for characterizing the functional state of the molecule. However, observations of the interaction between a monovalent cation and the polypeptide by $^{23}$Na NMR are a more appropriate means of characterizing the channel state of gramicidin. While this reversible interaction between cation and polypeptide does not prove that gramicidin is conducting cations across the lipid bilayer, it does allow for a distinction between states of the gramicidin which either do and do not interact with cations. For this report, the ion-interacting state is considered to be that of the channel state. Finally, the use of CD, which is limited to low light scattering solutions, is further limited to a qualitative spectral fingerprint, because the assignment of the spectral lines is compromised by severe overlap of individual lines. Despite this latter limitation CD is very sensitive to certain structural changes and therefore can be used to good advantage. However, $^{23}$Na NMR is a much more appropriate technique for characterizing the functional state of gramicidin in planar lipid bilayers.

An example of the sensitivity of CD to structural differences is displayed by this work. The CD spectra obtained from gramicidin samples prepared out of trifluoroethanol and chloroform/methanol are very different. The only chromophores present in gramicidin which could be used to account for the spectral differences are the indole rings and the amide groups of the peptide backbone.

To account for such a major difference in the spectra either the backbone conformations of these two preparations are dramatically different or a substantial difference in the relative orientation of the indole rings exist. For these two preparations the solid state $^{15}N$ NMR spectra of oriented samples are remarkably similar. Most important is the similarity between the spectra in the region between 205 and 150 ppm, where the resonances are assigned to the polypeptide backbone. Apparently, there are at least six sites in the backbone that possess the same conformation and dynamics in the two preparations. Furthermore, there are many other resonances which cannot all be assigned to the indole rings that do not vary in chemical shift. Consequently, it can be stated that most of the backbone peptide linkages have the same orientation in these two preparations. Therefore, the torsion angles and hence the conformation of the backbone is identical for these three preparations within experimental error. Finally, it has been argued that the negative ellipticity near 230 nm is due to the tryptophan sidechains as is much of the ellipticity below 210 nm (Urry, 1985; Masotti et al., 1980). Therefore, the most likely explanation for the CD results is a conformational rearrangement of the tryptophan sidechains. How large a rearrangement and how many of these sidechains are needed to explain the observed spectral changes is not known.

In the solid state $^{15}N$ NMR spectrum the resonances from the indole $^{15}N$ sites are expected between ~150 and 40 ppm. No prominent differences occur in this region of the three $^{15}N$ NMR spectra. Clearly, the NMR signals for the different tryptophan sidechain conformations suggested by the CD results are not resolved in this complex region of the spectrum where powder pattern intensity as well as many other amide resonances overlap. The lack of resolution may result from the indole resonances being broad due to motions that are slow on the NMR timescale ($10^4$ Hz) or due to short $T_2$ relaxation times. Alternatively, the lack of resolution may be due to poor sensitivity from inefficient cross-polarization (a result of unfavorable relaxation times); or the poor sensitivity could be due to the resonance from a single indole ring being split into several lines by the occurrence of several conformational substates. Answers to such related questions will have to await studies of single-site indole-labeled gramicidins. In fact, sidechain conformational states (Urry et al., 1981; Urry, 1984) have been suggested as the cause of the variability in the conductance states observed for gramicidin (Bamberg et al., 1976; Busath and Szabo, 1981; Busath et al., 1987).

These $^{15}N$ spectra of uniformly $^{15}N$ labeled gramicidins do not show the anticipated differences in tryptophan sidechain orientations, which result from the solvent history dependence. However, these spectra do clearly show that there is no global structural difference between the channel and nonchannel states as would be necessary to explain the CD spectra based on the amide chromophores. Consequently, the CD and $^{15}N$ NMR spectra of these gramicidin states, as characterized by $^{23}Na$ NMR, can be explained by differences in the tryptophan sidechain orientations.

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