Quast, U., Engel, J., Steffen, E., Tschesche, H., Jering, H., & Kufer, S. (1975) *Eur. J. Biochem. 52*, 511-514.
Ramachandran, G. N., & Mitra, A. K. (1976) *J. Mol. Biol. 107*, 85-92.
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764-4768.
Sherman, M. P., & Kassell, B. (1968) *Biochemistry 7*, 3634-3641.
Snow, J. W., Hooker, T. M., Jr., & Schellman, J. A. (1977) *Biopolymers 16*, 121-142.
Snyder, G. H., Rowan, R., III, Karplus, S., & Sykes, B. D. (1975) *Biochemistry 14*, 3765-3777.
Snyder, G. H., Rowan, R., III, & Sykes, B. D. (1976) *Biochemistry 15*, 2275-2283.
States, D. J., Dobson, C. M., Karplus, M., & Creighton, T. E. (1980) *Nature (London) 286*, 630-632.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113-174.
Strickland, E. H., Wilchek, M., Horwitz, J., & Billups, C. (1970) *J. Biol. Chem. 245*, 4168-4177.
Strickland, E. H., Wilchek, M., Horwitz, J., & Billups, C. (1972) *J. Biol. Chem. 247*, 572-580.
Takagi, T., & Ito, N. (1972) *Biochim. Biophys. Acta 257*, 1-10.
Vincent, J.-P., Chicheportiche, R., & Lazdunski, M. (1971) *Eur. J. Biochem. 23*, 401-411.
Wagner, G., & Wüthrich, K. (1975) *J. Magn. Reson. 20*, 435-445.
Wagner, G., Tschesche, H., &. Wüthrich, K. (1979a) *Eur. J. Biochem. 95*, 239-248.
Wagner, G., Kalb (Gilboa), A. J., & Wüthrich, K. (1979b) *Eur. J. Biochem. 95*, 249-253.
Woody, R. W. (1978) *Biopolymers 17*, 1451-1467.
Wüthrich, K., & Wagner, G. (1979) *J. Mol. Biol. 130*, 1-18.

Conformation of Gramicidin A in Phospholipid Vesicles: Circular Dichroism Studies of Effects of Ion Binding, Chemical Modification, and Lipid Structure†

B. A. Wallace,* W. R. Veatch, and E. R. Blout

ABSTRACT: The effects of cation binding, chemical modification, and lipid structure on the conformation of the channel-forming polypeptide gramicidin A in phospholipid vesicles have been investigated by circular dichroism spectroscopy in order to aid in elucidating the mechanism of action of this antibiotic and to ascertain features necessary for maintenance of its structure in membranes. Gramicidin A is capable of adopting a number of different conformations, depending on its environment. In organic solvents, it forms a family of double-helical dimers, whereas in membranes, it forms an N-terminal to N-terminal dimer of single helices. None of the structures in a variety of hydrophilic and amphipathic organic solvents are equivalent to its structure in membranes. A mechanism for cation binding in which the channel widens (and consequently the helix foreshortens) had been proposed on the basis of studies of crystals formed from organic solvents. This study of gramicidin demonstrates that in membranes the helical pitch (and, therefore, width and length) of the molecule remains unaltered upon binding of ions and suggests that the ion channel binding mechanism must involve only small, local changes such as position of side chains or hydrogen bonds near the cation binding site. The effect of the membrane lipid thickness and organization on peptide conformation has been examined by altering fatty acid chain lengths and the lipid phase state, respectively. In addition, variations of peptide–lipid ratios indicate that peptide–lipid interactions must be important in maintaining the helical conformation. Conformational studies of chemically modified gramicidin molecules have been correlated with their conductance properties.

Gramicidin A[1] is a linear polypeptide antibiotic consisting of 15 hydrophobic amino acids of alternating L and D configuration, whose primary structure has been determined (Sarges & Witkop, 1965). It forms ion channels in membranes that are specific for small monovalent cations (Hladky & Haydon, 1972). Conductance and fluorescence measurements have demonstrated that the conducting species in membranes is the dimer (Veatch & Stryer, 1977; Bamberg & Lauger, 1973). The detailed structure of that dimer has been under investigation for a number of years. Urry first proposed that it consisted of two end-to-end single helices with a β-sheetlike hydrogen-bonding pattern, which he denoted π (L,D) (Urry, 1971). That the N-terminal to N-terminal dimer was the major form adopted in black lipid films was strongly suggested by conductance measurements using modified gramicidins (Bamberg et al., 1977; Bradley et al., 1978; Szabo & Urry, 1979). A minor component consisting of C-terminal to C-terminal dimers was later also considered to be a possibility

† From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032 (B.A.W.), and the Departments of Biological Chemistry (E.R.B) and Pharmacology (W.R.V.), Harvard Medical School, Boston, Massachusetts 02115. *Received October 28, 1980; revised manuscript received May 14, 1981.* This work was supported by U.S. Public Health Service Grant GM-27292 and Jane Coffin Childs Faculty Grant-in-Aid 359 to B.A.W., AI-15311 and RCBA AI 00320 to W.R.V., and AM 01300 and AM-10794 to E.R.B. This project was supported in part by Biomedical Research Support Grant 507 RR05395 to Columbia University, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. B.A.W. is the recipient of a Hirschl Career Scientist Award.

[1] Abbreviations used: CD, circular dichroism; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; DMPC, dimyristoylphosphatidylcholine; gramicidin A, a mixture of 85% gramicidin A, 10% gramicidin B, and 5% gramicidin C.

0006-2960/81/0420-5754$01.25/0

(Bradley et al., 1978). In contrast, Veatch et al. (1974), on the basis of CD and IR spectroscopic studies of the molecule in organic solvents, proposed that the dimer formed intertwined double helices (with either parallel or antiparallel strands). Both the single- and double-helical models could result in channels with a pore diameter of ~3–4 Å and a channel length of ~30 Å. To ascertain whether either of these structures actually obtained in membrane bilayers, NMR studies were done by using specifically labeled gramicidins and indicated that the N-to-N single-helical dimer existed in phospholipid vesicles (Weinstein et al., 1979, 1980). In this work, the relationship between the structure formed in membranes and the structure formed in organic solvents has been examined by circular dichroism in order to determine if the discrepancy in the previously reported results is due to the polymorphic nature of the molecule in the various "solvent" systems or to difficulties in interpretations of the previous data, and whether any organic solvent system could be found in which the molecule retained the same conformation as in membranes.

X-ray crystallographic studies by Koeppe et al. (1978) indicated that the structure of gramicidin in crystals formed from methanol and ethanol is a helical channel with a diameter around 5 Å and a length of 32 Å. These workers have not yet distinguished whether the structure is a double helix or two single helices. In their crystals, the channel becomes shorter and wider upon binding of cesium (Koeppe et al., 1979). However, it may be that the structure in these crystals more closely resembles the structure of the molecule in organic solvents (from which it has been formed) rather than the structure in the membrane. Since the channel is specific for monovalent cations, the present circular dichroism study examines the effect of cesium binding to the channel in membranes for two purposes: to examine the mechanism of cation binding and to demonstrate the relation of the crystal structure to the membrane-embedded structure.

Studies using black lipid films have demonstrated that as the membrane thickness increases, gramicidin single channel conductance remains constant but that the mean channel lifetime decreases, suggesting a decrease in the dimerization constant (Hladky & Haydon, 1972; Veatch et al., 1975; Kolb & Bamberg, 1977). It has been proposed that the lipids in the thicker membranes must dimple in to accommodate a constant channel length, resulting in an energy requirement for the distortion of 0.1 kcal/Å (Urry et al., 1975). NMR studies have examined the effect of gramicidin on the ordering of the lipid molecules and have suggested the absence of a specialized boundary area in contact with the peptide, using very high peptide–lipid ratios (Oldfield et al., 1978). This is at variance with ESR and X-ray studies using lower peptide concentrations (Chapman et al., 1977). The relevance of peptide–lipid ratios in maintaining structural integrity of the channel has not been previously determined. For an examination of any effects of membrane thickness, lipid phase states, and peptide–lipid interactions on the conformation of gramicidin in membranes, CD studies using phospholipids with different fatty acid chain lengths, various temperatures, and altered peptide–lipid ratios have been done in this study.

Finally, certain modifications of the polypeptide, such as removal or alterations of the N terminus, result in decreased conductance activity (Morrow et al., 1979), the structural ramifications of which have also been examined in this spectroscopic study.

Materials and Methods

Synthesis of Gramicidin Derivatives and Analogues. Gramicidin A from ICN was crystallized once from ethanol. Desformylgramicidin (desF), desformylvalylgramicidin (desFV), and *O*-acetylgramicidin (O-Ac) were prepared as previously described (Weinstein et al., 1979), using procedures modified from those of Sarges & Witkop (1965).

N,O-Diacetyldesformylgramicidin was prepared by acetylation of desformylgramicidin using 8-fold excess of acetic anhydride in pyridine at 37 °C for 24 h. After evaporation to dryness, methanol was added and the solution reevaporated to remove any remaining pyridine. *N*-Acetyldesformylgramicidin (N-Ac) was prepared from *N,O*-diacetyldesformylgramicidin by hydrolysis with 2 N KOH in methanol for 20 h, followed by purification on a cation-exchange column AG-MP-50 (Bio-Rad) (11 × 4 mm) eluted with methanol. The purity and identity of these compounds were assessed by NMR and high-pressure liquid chromatography with methods previously described (Weinstein et al., 1979).

Preparation and Characterization of Vesicles. Sonicated vesicles were prepared as previously described (Wallace & Blout, 1979) by using peptide/lipid ratios varying from 1:3 to 1:40. All vesicles were prepared and sonicated at temperatures above their lipid phase transition temperature. Unless otherwise noted, lipid concentrations of 4 mg/mL and peptide/lipid ratios of 1:30 were utilized in the spectroscopic studies.

For salt titrations, vesicles were prepared as usual, except that the total vesicle concentration was initially 2-fold greater than normal. The salt solutions (2 × final concentration) or H_2O was added to the vesicles (1:1 v/v) and the suspensions were resonicated.

For methanol titrations, methanol was added slowly with stirring to the vesicles to prevent high local methanol concentrations within the sample.

Vesicles were prepared for microscopy as follows: 10 μL of vesicle suspension (1 mg/mL lipid) was placed on a Formvar coated copper grid for 2 min, and the grid was washed with 10 μL of deionized water and stained with 1% aqueous uranyl acetate for 15 s. Grids were examined in a Philips 301 electron microscope, the magnification of which had previously been calibrated with tobacco mosaic virus.

Conductance measurements were performed on glycerol monooleate and tetradecane planar bilayer membranes as previously described (Weinstein et al., 1980; Morrow et al., 1979).

Circular Dichroism Measurements. CD spectra of freshly prepared samples were recorded on a Cary 60 spectropolarimeter with a Model 6001 CD attachment and a variable position detector operating over the wavelength range 300–190 nm. A temperature-controlled sample cell of 1-mm path length was used.

Blank runs of vesicles in H_2O or vesicles in the corresponding salt solutions were subtracted from the measured spectra to obtain the spectra of the gramicidin molecules. The spectra reported are the average of three scans from at least two independent preparations for each sample.

For an assessment of the light-scattering properties of the vesicles, the variable position detector was utilized. When the sample cell was located at various distances from the photomultiplier, a number of different acceptance angles were obtained. For most experiments, the cell was located adjacent to the photomultiplier, resulting in maximum light acceptance.

For a number of experiments, UV spectra of vesicles and vesicles solubilized in 1% $NaDodSO_4$ were obtained over the wavelength range 300–190 nm in order to determine the extent of light scattering and the necessity for pseudo-reference-state

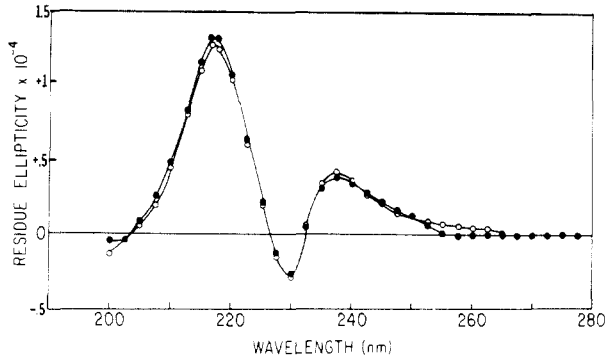

FIGURE 1: CD spectrum of gramicidin A in DMPC vesicles at 20 °C in the presence (○) and absence (●) of 2 M CsCl.

correction (Urry, 1972). For the specimens reported, such corrections were found to be insignificant and thus were not applied to the data.

The sample temperature was regulated with an external circulating bath and monitored by using a thermistor probe.

Results

Characterization of Samples. The sonicated lipid vesicle specimens used in this study were shown by negative stain electron microscopy to be ~300 Å in diameter regardless of whether they contained gramicidin molecules. NMR studies indicated that essentially all the gramicidin molecules partition into the membrane (Weinstein et al., 1980) and that no detectable amount was located in the surrounding aqueous phase under conditions used to prepare these vesicles.

One possible artifact that must be considered in doing spectroscopic studies of particulate samples is the potential light scattering by the specimens. This could result in peak shifts to longer wavelengths and absorption flattening effects which could cause a decrease in total amplitude (Urry, 1972). These effects have been correlated with particle size (Litman, 1972) and were shown to be insignificant for small vesicles in this concentration range (Wallace & Blout, 1979). Any such effects may be minimized by using a short specimen-to-detector distance and a short path length cell (Schneider & Harmetz, 1976). The amount of light scattering from the translucent vesicles used in this study was negligible, as demonstrated in spectra obtained at various acceptance angles using different specimen-to-detector distances, and by calculation of the magnitude of pseudo-reference-state corrections (Urry, 1972) (~1%). There was no apparent change in the light-scattering properties of the vesicles with lipid state, thus permitting experiments to be done which examined the gramicidin structure above and below the lipid phase transition temperature. Vesicle contributions to the spectra were very small and were subtracted by use of a blank containing vesicles without peptide present.

Solvent Effects. The CD spectrum of gramicidin in dimyristoylphosphatidylcholine vesicles (Figure 1) has a λ_{max} at 217 nm with a residue ellipticity equal to $+1.3 \times 10^4$ and a λ_{min} at 229 nm with an ellipticity of -0.3×10^4 as well as a secondary maximum of ellipticity 0.38×10^4 at 237.5 nm (although the magnitude of this peak is slightly variable between preparations) (Figure 1). This spectrum differs completely (in θ, λ_{max}, and λ_{min}) from the spectrum of gramicidin in methanol as well as from the spectra obtained in dioxane, ethanol, and 2-propanol solutions (Veatch et al., 1974) for the various gramicidin dimer conformers.

Attempts were made to find a solvent which would more nearly approximate the hydrophobic interior of the bilayer. Studies with a small peptide which was completely buried within the bilayer interior have suggested that the linear hydrocarbons hexane and hexadecane provide a good approximation of the environment of the bilayer (Wallace & Blout, 1979). However, gramicidin, which spans the membrane and must interact with both the hydrophobic and hydrophilic portions of the bilayer, exhibited too low a solubility in these solvents to be useful for spectroscopic studies. In this study, amphipathic solvents having hydrophobic and hydrophilic ends, such as decanol (which has been proposed to be thermodynamically equivalent to the bilayer) and decanoic acid, resulted in spectra similar to those found for the shorter chain alcohols, but totally unlike the vesicle spectrum (data not shown).

Whether the presence of lipid might be necessary to induce the gramicidin to adopt its "membrane" conformation was investigated by using methanol solutions of gramicidin and lipid. However, the mere addition of lipid (at a lipid/peptide ratio of 31:1) was not sufficient; the spectra obtained were essentially identical with those for methanol solutions of gramicidin alone (data not shown). This experiment suggested that perhaps an ordered juxtaposition of hydrophobic and hydrophilic regions, rather than a homogeneous solution, was essential to maintain the conformation. Therefore, spectral studies of the solubilization of preformed vesicles were done. Vesicles containing lipid and gramicidin were slowly titrated with methanol, and spectra were taken of samples containing 1%, 2%, 5%, 10%, 20%, 40%, 60%, and 80% methanol. The spectrum remained unchanged up to and including the addition of 40% methanol. Above this concentration, the spectrum was essentially identical with the spectrum in 100% methanol. Above 40% methanol, the vesicles appeared to dissolve, forming a homogeneous solution, and this observation was confirmed as being the point at which vesicular structures were no longer detected by electron microscopy. Similarly, when water was added to a solution of gramicidin and lipid in methanol, at less than 40% methanol, lamellar-like structures formed, and the spectrum reverted to the "membrane" form. These results seem to indicate that gramicidin required the ordered matrix of the lipid bilayer rather than simply the presence of hydrophobic and hydrophilic components to form the membrane-associated structure.

Ion Binding Effects. One suggestion for the mechanism of cation binding in the gramicidin channel has been that the dimer widens and foreshortens on ion binding (Koeppe et al., 1978). A consequence of such a conformational change would be a change in the pitch of the helix (Figure 2), which should be detectable by CD. However, the CD spectra of vesicles containing gramicidin and either CsCl or NaCl showed no change (Figure 1) over the ion concentration range 0–2.0 M. At these concentrations, the monovalent cation binds in the channel and should be sufficient to saturate all the binding sites (Hladky & Haydon, 1972). Spectra were obtained from samples titrated with 0.1, 0.5, 1.0, and 2.0 M cesium ions. The ellipticity values, λ_{max}, λ_{min}, and the secondary λ_{max} were identical in all samples within the precision limit of ±2%. The very small difference in θ at long wavelengths (~260 nm) at high cesium concentrations could possibly arise from small changes in side-chain conformation, resulting in a small change in the tryptophan transition. Also, sodium ion concentrations of 0.5 and 2.0 M resulted in the same spectra as those without this ion. The absence of a significant change in ellipticity indicates no major change in helical pitch (and, therefore, channel length and width) or overall peptide conformation occurring in these membranes.

Effect of Lipid State and Structure. For determination of the effect, if any, of lipid organization and fluidity on the

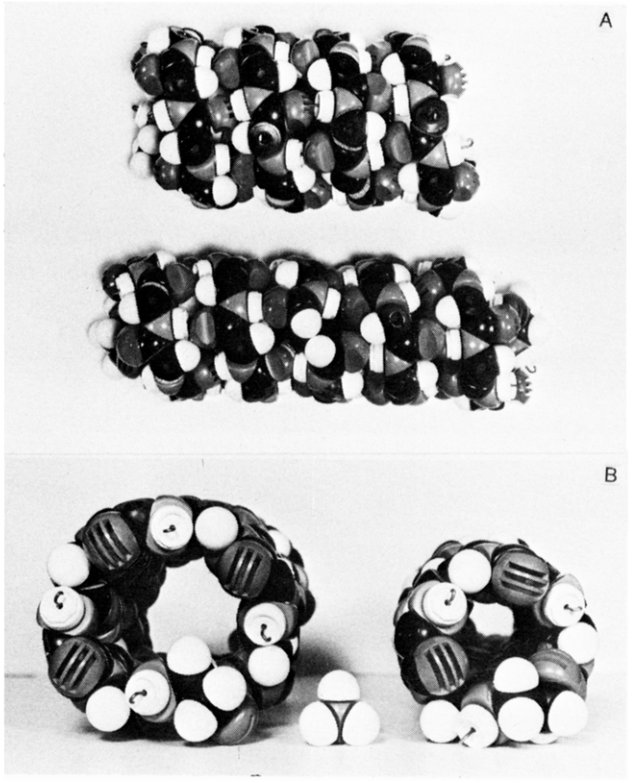

FIGURE 2: Gramicidin molecular model (CPK). (A) Side view of single helix dimer (without side chains, for clarity) of Π_8(L,D) and Π_6(L,D) helixes. (B) Top view of single helix dimers of Π_8(L,D) and Π_6(L,D) helixes showing ion channel.

structure of gramicidin in membranes, gramicidin was incorportated into DMPC vesicles, and CD spectra were obtained at a number of temperatures above, below, and at the lipid phase transition. For DMPC, the middle of the main phase transition is 23 °C (Janiak et al., 1976). Even if the transition is broadened substantially by the presence of peptide (Chapman et al., 1977) (which could be as much as ±7 °C), the extreme values of 4 and 50 °C are clearly outside the transition range and represent lipids in gel and liquid-crystalline phases, respectively. Light scattering was insignificant even at temperatures below the phase transition temperature. The gramicidin spectra obtained at all these temperatures in vesicles were identical within the precision limit of ±2% (data not shown). This indicates that the fluidity of the lipid fatty acid chains has no detectable effect on peptide conformation, at least for these short lipid chains, nor does the change in bilayer thickness from 44 to 35 Å (Janiak et al., 1976) affect the peptide structure.

It is possible that a change in bilayer thickness with longer lipid chain lengths which are not compatible with the gramicidin dimer length may have an effect on conformation. For further identification of any bilayer thickness effect, vesicles were prepared from lipids of different length saturated fatty acid chains: dilaurylphosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine. Below the phase transition temperatures, each successive addition of two methylene groups results in about a 5 Å increase in bilayer thickness (Janiak et al., 1976). Thus the experiments utilized bilayers of thickness ranging from 39 to 54 Å.

A family of curves (Figure 3) is obtained for native gramicidin in bilayers of different thicknesses. For the very thin membranes, dilauryl- and dimyristoylphosphatidylcholines, there is little difference in the spectra. However, as the thickness increases, with dipalmitoyl- and distearoyl-

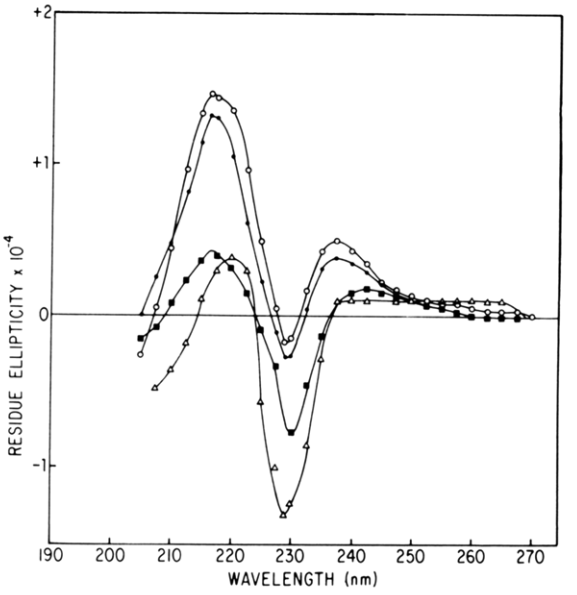

FIGURE 3: CD spectrum of gramicidin A in (○) DLPC, (●) DMPC, (■) DPPC, and (△) DSPC at 20 °C (lipid/peptide 30:1).

Table I: Conductance Properties of Gramicidin Analogues

analogue	relative steady-state conductance
gramicidin	1.0
O-acetylgramicidin	1.2
N-acetyldesformylgramicidin	0.11
Desformylgramicidin	0.002
Desformylvalylgramicidin	0.0001

phosphatidylcholines, the positive ellipticities at 237 and 217 nm decrease, and the negative ellipticity at 229 nm increases. The λ_{max} and λ_{min} are unaffected.

Effect of Peptide–Lipid Interactions. For determination of the importance of peptide–lipid and peptide–peptide contacts in maintaining gramicidin structure in membranes, the relative concentrations of gramicidin and DMPC were varied. At low peptide–lipid ratios [1:15, 1:30, and 1:45 (m/m)], the spectra were essentially identical. However, at very high ratios (i.e., 1:1), the material did not form vesicular-like structures, and the resulting suspensions scattered light considerably. At the highest ratios achieved without excessive light scatter (1:3 and 1:4), the CD spectra were significantly distorted: they consisted of a single negative peak with a mean residue ellipticity of 0.99×10^3 at 235 nm. Clearly, absorption flattening is responsible for the low ellipticity and possibly the shift of the absorption band. But the absence of a large positive band at lower wavelengths suggests that a different gramicidin structure produces this spectrum and indicates that these high protein concentrations [which would result in virtually close-packed peptide and regions of peptide–peptide contact without intervening lipid molecules (Chapman et al., 1977)] are disruptive and that peptide–lipid interaction is important.

Effect of Chemical Modification. For examination of whether chemical modifications which lead to changes in conductance properties can be detected as changes in molecular conformation, CD studies of modified gramicidin molecules incorporated into vesicles were performed (Figure 4). Gramicidin which has been modified at the C terminus with an acetyl group (O–Ac) is a fully active molecule (Table I). This modification at the C terminus does not interfere with N–N dimer formation, so the conformation of this channel is expected to be like native gramicidin in lipid vesicles. The only real difference in the spectra of the two molecules is a

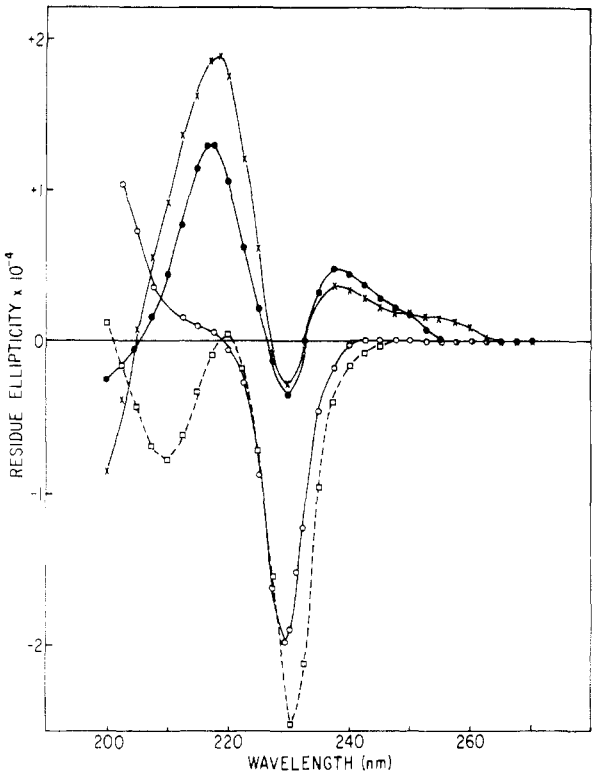

FIGURE 4: CD spectrum of *O*-acetyl- (●), *N*-acetyl- (×), desformyl- (○), and desformylvalylgramicidins (□) in DMPC vesicles at 20 °C.

20% increase in the θ of the secondary maximum at 237 nm. Gramicidin which has been modified at the N terminus by replacing the formyl group with an acetyl group (N-Ac) has reduced activity but still forms a reasonably large number of dimer channels. In this case, the acetyl group does not hydrogen bond or pack so compactly in the center of the bilayer. The spectrum of N-Ac gramicidin exhibits the same general features as native gramicidin, with the only significant differences being a 50% increase in the θ of the λ_{max} at 217 nm, suggesting a slight difference but generally the same channel conformation. Two other modifications at the N terminus, desformyl- and desformylvalylgramicidin, exhibit a nearly complete loss of activity that is likely due to their inability to form dimers (Morrow et al., 1979). The CD spectra of these two molecules in lipid bilayers are vastly different from those of native gramicidin in lipid bilayers. Their negative ellipticity at 231 nm (a peak shift of ~2 nm) is nearly an order of magnitude larger than the native molecule, and the positive band that peaked at 217 nm is absent. These spectra may be a result of a combination of conformations which are inactive; however, the contribution of any dimer similar to native must be very small indeed, if any. In fact, these spectra are more reminiscent of the nonmembrane conformation of the native molecule in organic solvents.

Discussion

The goals of this CD study were to examine the structure of gramicidin as it is found in membranes and to define changes which the molecule undergoes under various physiologically important conditions in order to elucidate its mechanism of action. Further studies are underway to determine more detailed information about the gramicidin structure in membranes, such as helical pitch by X-ray diffraction of oriented, hydrated bilayers (B. A. Wallace, unpublished results) and of three-dimensional crystals formed under condtions which mimic the bilayer [i.e., containing lipid molecules (B. A. Wallace, unpublished results)].

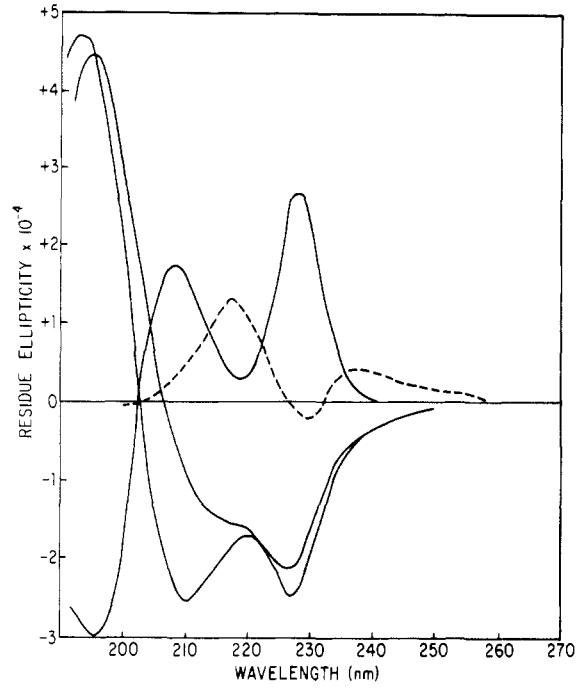

FIGURE 5: CD spectra of (—) isolated conformers in 2-propanol solution or dissolved crystals observed under minimum interconversion conditions [from Veatch et al. (1974)] and (---) in phospholipid vesicles (this work).

As shown in this work, it is quite clear that the conformation gramicidin adopts in membranes is not the same conformation as any of those conformations adopted in organic solvents (Veatch et al., 1974) and may not be the form it adopts in the crystals prepared from alcohol solutions (Koeppe et al., 1979). For this polypeptide which spans the membrane, organic solvents are not good models for the lipid bilayer. The spectra of gramicidin obtained in a number of alcohols (methanol, ethanol, 2-propanol, 1-octanol, and 1-decanol) as well as in dioxane and decanoic acid solutions were totally different than those obtained in lipid vesicles (see Figure 5 for comparison). The various spectra in organic solvents have been proposed to arise from a family of double-helical structures (Veatch et al., 1974). The vesicle spectrum cannot be produced by any linear combination of the spectra of these conformers. Assignment of a specific channel structure based solely on the gramicidin CD spectrum in vesicles is difficult. This spectrum is not reminiscent of any known polypeptide structures. Interpretation is complicated by the high concentration of aromatic side chains and by the presence of L- and D-amino acids, which result in strong interactions among tryptophan transitions and with peptide backbone transitions. However, the structures formed in organic solvents are obviously not the same as the channels formed in vesicles. That the conformation of this molecule in membranes and in organic solvents is different is reasonable since these sytems exhibit very different hydrogen-bonding capabilities and geometries. Molecules such as gramicidin which span the bilayer may require a juxtaposition of polar and nonpolar regions. The mere presence of an amphiphilic molecule (phospholipid) in the alcoholic or aqueous/alcoholic solutions was not sufficient to convert the gramicidin conformation to the membrane-bound form but rather required an ordered arrangement of hydrophobic and hydrophilic regions in a bilayer.

The only other system which appears to permit the gramicidin to adopt a similar conformation to the channel in membranes may be lysophosphatidylcholine micelles which would also impose a one-dimensional organization on the

molecule. Masotti et al. (1980) have reported spectra for gramicidin incorporated by heating into micelles as well as egg lecithin liposomes, which have some features similar to those observed in the spectra reported here, again supporting the requirement for an ordered matrix.

Previously NMR studies of gramicidin in vesicles (Weinstein et al., 1980) and CD and IR studies of gramicidin in organic solvents (Veatch et al., 1974) had suggested different structures for the dimer. This work supports both these studies as being correct. The different results obtained arise because different species were being examined; the molecular structures formed in vesicles and in organic solutions are not identical.

In membranes, gramicidin does not undergo a major conformational change that involves alteration of the pitch of the helix or the width of the channel upon binding ions. On the basis of the changes in the channel size in crystals grown in methanol in the presence of cesium ions, compared with those grown in the absence of the ions, Koeppe et al. (1980) proposed that the channel widens on binding cations to permit carbonyl–metal coordination and implied (Koeppe et al., 1979) that it is a mechanism for ion binding of the channel in membranes. A consequence of such a conformational change would be a change in the pitch of the helix (Figure 2), which should be detectable by CD. While exact calculations for gramicidin A are complicated by the high tryptophan content of the molecule, it is reasonable to expect a decrease on the order of 10–50% if the sort of change suggested by the crystals is indeed occurring. Even if the conformation in the membrane is something other than a Π(L,D) helix, any significant conformational change on binding ions would be detected as a change in the CD spectrum. The CD results reported here indicate that the spectra are unchanged in the presence of saturating concentrations of monovalent cations. These results indicate no change in helical pitch, and thus no widening of the channel and foreshortening of the molecule occur in the membrane-type conformation. This result is reasonable since the radius of the cesium ion (even in its fully hydrated form) is only 2.28 Å (Cotton & Wilkinson, 1966). The π_6(L,D) helix has a channel diameter of ~4 Å (Urry et al., 1971), so an ion could clearly be accommodated in the channel without requiring the channel to widen (Figure 2). Possibilities for why such an increase in diameter occurred in the crystals and not in membrane include the following: (1) organic solvents occupy space in the crystal channel and are not displaced by the ion, thus resulting in insufficient room for ions to bind without widening, or (2) a different structure is found in crystals (i.e., a double helix), which behaves differently in the presence of ions than does the single-helical dimer form of membranes. This latter suggestion may be supported by the observations that binding of cations to gramicidin in organic solvents causes a substantial change in the CD spectrum and thus the conformation of the molecule (B. A. Wallace, unpublished observation; Veatch, 1973). This suggests that the structure in crystals in the absence of lipids may not be equivalent to the structure in membranes and may more nearly reflect the conformation of the molecule in the solvent system from which it was crystallized.

The lipid requirements for obtaining functional lipid–gramicidin structures are relatively nonspecific, but very high peptide–lipid ratios or very thick membranes can cause changes in the peptide structure and its ability to dimerize. In DMPC vesicles, the structure as detected by CD is identical when the vesicle are examined at temperatures above, below, and at the lipid phase transition, so they do not require bulk fluid lipids to maintain their integrity. For the short chain lipids, the accompanying change in bilayer thickness with lipid state has little effect, most likely indicating that the gramicidin molecule does not elongate to accommodate the thicker bilayer (an action which would have been reflected as a decrease in the negative ellipticity). This invariant gramicidin length is consistent with the model (Hladky & Haydon, 1972) that proposes the surrounding lipid molecules dimple in to accommodate a constant peptide length rather than the peptide elongating to fit in a thicker lipid bilayer. With thicker bilayers (dipalmitoyl- and distearoylphosphatidylcholines), changes in the spectrum occur. Two possible explanations for these changes can be considered: (1) a change in the pitch of the helices or (2) a change in dimerization constant. We favor the latter as being more consistent with all the available data. The CD results argue against a change in helix pitch because a decrease in negative ellipticity would be predicted for a decreased number of residues per turn (Woody & Tinoco, 1967). A molecule which elongates to accommodate a thicker bilayer would decrease the number of residues per turn and consequently should result in a decrease in negative ellipticity. The opposite result was observed upon increasing the bilayer thickness. If a change in pitch of the helix were responsible for the change in helical parameters in going from thinner to thicker membranes, the change would have to be from a longer to a shorter helix, resulting in an even greater discrepancy between peptide and bilayer dimensions and clearly contrary to expectations. An alternative explanation would arise if thick membranes substantially destabilize the relatively short transmembrane dimer channel but do not alter the energy of a monomer which does not span the membrane. Consequently, the spectral change could represent an increasing contribution of monomer conformation(s) and spectra arising from molecules in several different inactive, nonconducting conformations. If more of the active species are present in the thinner membranes (as suggested by conductance measurements), then the dilaurylphosphatidylcholine spectrum may represent a mixture with a large proportion of the conducting species, while the thicker distearoylphosphatidylcholine spectrum would be due to predominantly nonconducting species. The intermediate thickness bilayers may be fractional sums of these two extremes. One cannot, from this work, absolutely exclude the possibility of change in either helix pitch or dimerization constant. However, by analogy with derivatives whose conductance properties are known, the more likely explanation for the changes seen in the spectra is due to a change in association constant to form active dimers. This explanation is also consistent with the results obtained by conductance studies using thicker membranes (Hladky & Haydon, 1972; Kolb & Bamberg, 1977; Veatch et al., 1975).

One additional benefit to doing these CD studies at various temperatures was to determine the validity of NMR studies done at high (52 °C) temperatures and low salt concentrations (Weinstein et al., 1979). This work shows that very similar conformations of the molecule must be present at all temperatures and that the channel is stable even in the absence of cations inside the channel.

Peptide–lipid interaction requirements for maintaining gramicidin channel structure were also reflected in studies using varying peptide–lipid ratios: at gramicidin/dimyristoylphosphatidylcholine ratios varying from 1:15 to 1:40, no changes were seen in the gramicidin spectrum. At these concentrations, peptide–lipid contact should be extensive (Chapman et al., 1977), and peptide–peptide interactions relatively rare (unless, of course, the gramicidin underwent a lateral phase separation into peptide-rich patches). This

latter possibility is unlikely, however, since at higher peptide concentrations (i.e., 1:3 and 1:4), totally different spectra result, suggesting a major alteration in peptide structure. At even higher peptide ratios, vesicular-like structures could not be formed. The different conformations at high concentrations, for which peptide–peptide contacts would be substantial and peptide–lipid contacts less significant, suggest that peptide–lipid interactions are important for maintaining gramicidin structures in membranes. It also indicates that other physical studies (Oldfield et al., 1978), which have used peptide–lipid ratios as high as 1:1, may not have been observing gramicidin structure in its native form.

The conductance activity of gramicidin in black lipid membranes can be correlated with spectral properties of the molecule in vesicles. Modifications at the C terminus, which did not substantially affect conductance (i.e., *O*-acetylgramicidin), yielded no change in the CD spectrum. This result also confirms that such a modified molecule [previously used in NMR (Weinstein et al., 1979) and conductance studies (Szabo & Urry, 1979)] reflects the native structure. Modifications of the N terminus (the portion embedded with the bilayer) were more substantive. Exchange of an acetyl group for the *N*-formyl group resulted in some decrease in activity but without total loss in the ability to form dimers (Szabo & Urry, 1979). This molecule produced an altered but generally similar spectrum to native, possibly resulting from altered helical structures. Modifications which destroyed conductance activity (i.e., desformyl or desformylvaline) produced vast changes in the CD characteristics. These spectra may result from the presence of several different conformers in the solution and may also reflect their decreased capacity to form the conducting dimer species.

In summary, these CD studies of gramicidin incorporated into small sonicated vesicles have provided insight into the conformation, mode of action, and membrane requirements for maintaining active channel structures.

References

Bamberg, E., & Lauger, P. (1973) *J. Membr. Biol. 11*, 177.

Bamberg, E., Appel, H. J., & Alpes, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2402.

Bradley, R. J., Urry, D. W., Okamoto, K., & Rapaka, R. (1978) *Science (Washington, D.C.) 200*, 435.

Chapman, D., Cornell, B. A., Eliaz, A. . & Perry, A. (1977) *J. Mol. Biol. 113*, 517.

Cotton, F. A., & Wilkinson, G. (1966) in *Inorganic Chemistry*, p 198, Wiley, New York.

Hladky, S. B., & Haydon, D. A. (1972) *Biochim. Biophys. Acta 274*, 294.

Janiak, M., Small, D., & Shipley, G. (1976) *Biochemistry 15*, 4575.

Koeppe, R., Hodgson, K., & Stryer, L. (1978) *J. Mol. Biol. 121*, 41.

Koeppe, R., Hodgson, K., & Stryer, L. (1979) *Nature (London) 270*, 723.

Kolb, H. A., & Bamberg, E. (1977) *Biochim. Biophys. Acta. 464*, 127.

Litman, B. (1972) *Biochemistry 11*, 3243.

Masotti, L., Spisni, A., & Urry, D. W. (1980) *Cell Biophys. 2*, 241.

Morrow, J., Veatch, W., & Stryer, L. (1979) *J. Mol. Biol. 132*, 733.

Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H. S., Hshung, J. C., Kang, S. Y., King, T., Meadows, M., & Rice. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4657.

Sarges, R., & Witkop, B. (1965) *J. Am. Chem. Soc. 87*, 2011.

Schneider, A., & Harmetz, D. (1976) *Biochemistry 15*, 4158.

Szabo, G., & Urry, D. (1979) *Science (Washington, D.C.) 203*, 55.

Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1907.

Urry, D. W. (1972) *Biochim. Biophys. Acta 265*, 115.

Urry, D. W., Goodall, M. C., Glickson, J. D., & Mayers, D. F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676.

Urry, D. W., Long, M. M., Jacobs, M., & Harris, R. D. (1975) *Ann. N.Y. Acad. Sci. 264*, 203.

Veatch, W. (1973) Ph.D. Thesis, Harvard University, Boston, MA.

Veatch, W., & Stryer, L. (1977) *J. Mol. Biol. 113*, 89.

Veatch, W., Fossel, E. & Blout, E. R. (1974) *Biochemistry 13*, 5249.

Veatch, W. R., Mathies, R., Eisenberg, M., & Stryer, L. (1975) *J. Mol. Biol. 99*, 75.

Wallace, B. A., & Blout, E. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1775.

Weinstein, S., Wallace, B. A., Blout, E. R., Morrow, J., & Veatch, W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4230.

Weinstein, S., Wallace, B. A., Morrow, J., & Veatch, W. R. (1980) *J. Mol. Biol. 143*, 1.

Woody, R. W., & Tinoco, I. (1967) *J. Chem. Phys. 46*, 4927.