STRUCTURE OF GRAMICIDIN A

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ABSTRACT Gramicidin A, a hydrophobic linear polypeptide, forms channels in phospholipid membranes that are specific for monovalent cations. Nuclear Magnetic Resonance (NMR) spectroscopy provided the first direct physical evidence that the channel conformation in membranes is an amino terminal-to-amino terminal helical dimer, and circular dichroism (CD) spectroscopy has shown the sensitivity of its conformation to different environments and the structural consequences of ion binding. The three-dimensional structure of a gramicidin/cesium complex has been determined by x-ray diffraction of single crystals using single wavelength anomalous scattering for phasing. The left-handed double helix in this crystal form corresponds to one of the intermediates in the process of folding and insertion into membranes. Co-crystals of gramicidin and lipid that appear to have gramicidin in their membrane channel conformation have also been formed and are presently under investigation. Hence, we have used a combination of spectroscopic and diffraction techniques to examine the conformation and functionally-related structural features of gramicidin A.

INTRODUCTION

Gramicidin A is a hydrophobic linear polypeptide antibiotic consisting of 15 amino acids with alternating L- and D-configurations. It has the sequence (Sarges and Witkop, 1965):

HCO-L-val-gly-L-ala-D-leu-L-ala-D-val-L-val-D-val-L-trp-D-leu-L-trp-D-leu-L-trp-D-leu-L-trp-$NHCH_2CH_2OH$.

In membranes, gramicidin A forms channels that are specific for monovalent cations (Hladky and Haydon, 1972); it is, to date, the physiologically best-characterized ion channel (Finkelstein and Andersen, 1981). Furthermore, it is an excellent model system for conformational studies of membrane proteins. Fluorescence and conductance measurements in black lipid membranes and phospholipid vesicles have shown that the active conducting form of the molecule is a dimer (Veatch et al., 1975; Veatch and Stryer, 1977). The structure of this dimer has been the subject of investigation in a number of laboratories.

In an early study, based, in part, on CD and NMR studies of the molecule in organic solvents, Urry first proposed (Urry, 1971; Urry et al., 1971) a π(L,D) helix conformation for the channel in which two helical monomers were associated in the membrane via their amino-termini (Fig. 1 *A*). Conductance studies using chemically modified gramicidins in black lipid films formed from glycerol monooleate and decane (Szabo and Urry, 1979; Bamberg et al., 1977) supported this model as well as, to some extent, a similar helical dimer with the carboxyl-termini associated instead (Bradley et al., 1978) (Fig. 1 *B*). Based primarily on IR and CD measurements of gramicidin in alcohol and dioxane solutions, Veatch et al. (1974) proposed an alternate family of models (Fig. 1 *C*, *D*), the parallel and antiparallel intertwined double helices. None of these studies, however, examined the structure of the molecule in phospholipid bilayers.

While the molecular folds of the helical dimer and double-helical models are completely different, they have many similar physical characteristics, making distinguishing between these structures difficult. In the double helix, the adjacent hydrogen bonds are intermolecular, while in the helical dimer, most are intramolecular. However, because the pitch of the double helix is twice that of the helical dimer, the chain separation per turn along the helix axis and the hydrogen bonding patterns are similar. Furthermore, the lengths (~30 Å), and outer (~15 Å) and inner channel diameters (~4–5 Å) of the two folding motifs are nearly indistinguishable. One striking difference between the two types of models is the location of their amino- and carboxyl-termini relative to the bilayer surface. Model *A* has the carboxyl-termini exposed at the surface and the amino-termini buried deep in the membrane. In contrast, model *B* has the carboxyl-termini buried and the amino-termini exposed. Models *C* and *D* have all their amino- and carboxyl-termini at the surface.

Hence, the gramicidin structure in membranes could be identified by a method that differentially probed locations normal to the bilayer surface. ^{13}C- and ^{19}F-NMR studies of gramicidin in phospholipid vesicles were able to make such distinctions (Weinstein et al., 1979; Weinstein et al., 1980). Using the membrane-impermeable probes Tm^{+++} and Mn^{++}, which are located in the aqueous space surrounding the bilayer, it was possible to show that the carboxyl-termini were exposed at the membrane surface

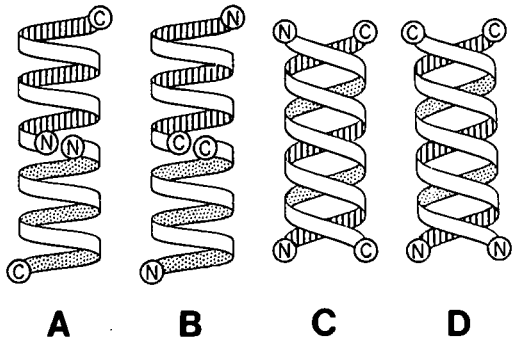

FIGURE 1 Schematic diagrams of models for the gramicidin A dimer: (*a*) amino-to-amino helical dimer; (*b*) carboxyl-to-carboxyl helical dimer; (*c*) antiparallel double helix; and (*d*) parallel double helix.

and that the amino-termini were inaccessible to the surface probes, which is consistent with model *A*. However, the negative finding concerning the amino-termini was not conclusive because it could have been a result of, for example, steric hindrance to accessibility. To clarify this issue, another paramagnetic probe, located on the distal end of a phospholipid molecule, was used to determine the location of sites in the membrane interior. This probe had the opposite effect of the surface probes: the amino-termini of gramicidin, but not the carboxyl-termini, were affected, thus showing that the amino terminal ends of the polypeptide are indeed buried in the lipid bilayer, while the carboxyl termini are surface exposed, consistent only with model *A*. This same sort of approach has been taken more recently by Urry et al. (1983*a*) to locate ion binding sites of gramicidin in detergent complexes, and to confirm this assignment of the orientation.

It is of interest to reconcile with these results the data based on studies in organic solvents that suggested the alternate family of double helical folding motifs. That is, does the molecule adopt the same conformation in isotropic, hydrophilic organic solvents and in anisotropic, hydrophobic lipid bilayers? Also, what are the detailed structures of the molecule in these two environments?

MATERIALS AND METHODS

Materials

Gramicidin (ICN Nutritional Biochemicals, Cleveland, OH), a mixture of 80% gramicidin A, 6% gramicidin B, and 14% gramicidin C, was crystallized from absolute ethanol and used without further purification. In this paper, this mixture will be refered to as gramicidin A. Gramicidin concentrations were determined using the molar extinction coefficient (ϵ = 22,000) at 282 nm. Lipid concentrations were determined by phosphate assay (Fiske and Subbarow, 1925). All salts and solvents used were reagent or spectrograde.

Sample Preparations

Type I Vesicles. Most vesicles were prepared as previously described (Wallace et al., 1981), by co-solubilization of gramicidin and dimyristoyl phosphatidylcholine (DMPC) in chloroform/methanol, followed by removal of solvent under vacuum, hydration in deionized water, and sonication above the lipid phase transition temperature. Peptide-to-lipid molar ratios ranged from 1:15 to 1:363.

Type II Vesicles. For the binding and insertion studies, gramicidin was added to preformed sonicated DMPC vesicles as previously described (Wallace, 1984).

For both types of vesicle preparations, control vesicles were prepared in the same manner without gramicidin present. All type I and II vesicles (both with and without gramicidin) were <500 Å in diameter, as shown by negative stain electron microscopy. The differential light scattering and absorption flattening effects of these particles are small and will not significantly distort the resulting spectra (Mao and Wallace, 1984).

Titrations

Stock solutions of gramicidin (16.5 mg/ml in methanol) were diluted with either 6 M LiCl or 110 mM CsCl plus the appropriate amount of methanol to a constant gramicidin concentration (1.5 mg/ml).

Crystallizations

Gramicidin/cesium crystals were prepared in a manner similar to that previously described (Kimball and Wallace, 1984), except that the form II crystals used for data collection were prepared by addition of 190 μl of CsCl solution (100 mM in methanol) and 60 μl of methanol to 50 μl of gramicidin solution (150 mg/ml in methanol).

CD spectroscopy

Spectra were collected on either a Cary 60 spectropolarimeter with a 6001 CD attachment or an Aviv (Lakewood, NJ) 60DS spectropolarimeter. Both instruments had a variable position detector. Spectra were obtained with the photomultiplier located directly adjacent to the sample cell, resulting in a 90° acceptance angle, to reduce scattering effects. Measurements were made at 25°C using 0.01 to 0.1 cm pathlength cells. At least three scans of two independent samples were collected and averaged for each specimen.

Diffraction

Preliminary characterizations of the crystals were done using Ni-filtered Cu K_α radiation from a Philips x-ray generator operating at 40 kv, 30 ma. 15° precession photographs were taken with a crystal-to-film distance of 100 mm. The high-resolution (1.8 Å) data set was collected on a single (0.16 × 0.16 × 0.1 mm) crystal at room temperature (16–19°C) using Ni-filtered Cu K_α radiation on a Picker FACS-1 diffractometer. Friedel pairs were collected successively in blocks of 20 reflections. Details of data collection and processing will be described elsewhere (Wallace and Hendrickson, manuscript in preparation).

RESULTS AND DISCUSSION

To compare the structures of gramicidin in two different environments, it was important to do parallel experiments using a single physical technique. Circular dichroism spectroscopy permits direct comparison between gramicidin in small unilamellar phospholipid vesicles and in solution. Veatch et al. (1974) had shown that several different interconvertible species are present in alcohol solutions and that each has a unique spectrum. The net spectrum of an equilibrium mixture of these species in methanol is shown in Fig. 2. Neither the net nor the individual spectra correspond to those obtained for typical α-helical or β-sheet secondary structures (Fig. 2, *A* & *B* insets). This is a consequence of two features of the gramicidin primary structure: the alternating L- and D-amino acids that result in different ϕ,ψ angles for the polypeptide backbone, which affect the peptide transitions; and the high tryptophan

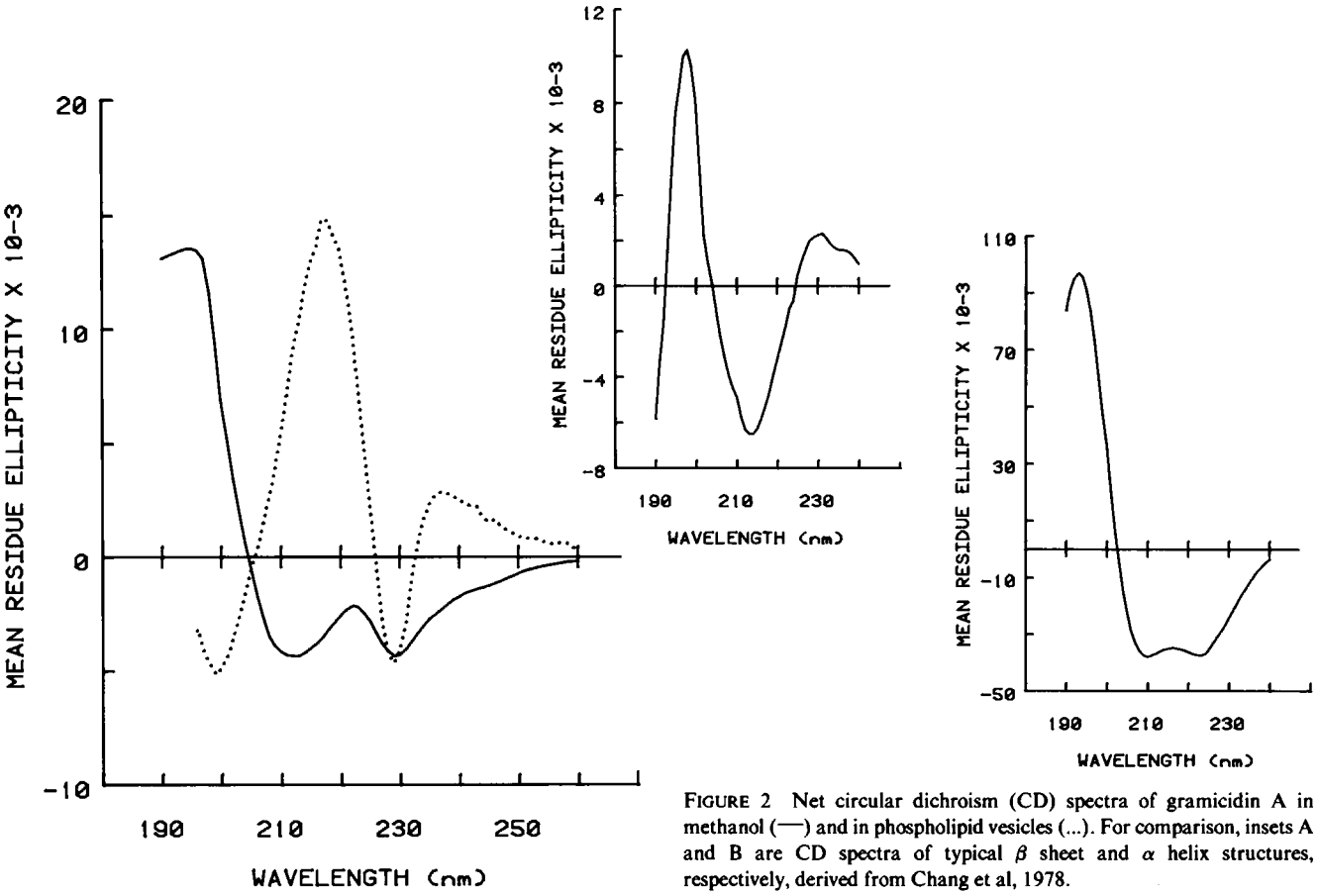

FIGURE 2 Net circular dichroism (CD) spectra of gramicidin A in methanol (—) and in phospholipid vesicles (...). For comparison, insets A and B are CD spectra of typical β sheet and α helix structures, respectively, derived from Chang et al, 1978.

content (4 out of 15 amino acids), for which the $\pi \rightarrow \pi^*$ transitions of the aromatic rings give rise to ellipticity at wavelengths normally dominated by the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the backbone. Unique structural assignments based on these CD spectra have not yet been made. Comparisons between spectra of the molecules in two environments are possible, however, and permit distinction between folding motifs. The spectrum of gramicidin in lipid vesicles (Fig. 2) is different from the net spectrum in organic solvents, and cannot be represented as a linear combination of any of the individual solution spectra (Wallace et al., 1981). Therefore, it must represent a distinct form of the molecule. The far ultraviolet (UV) portion of the membrane spectrum has an unusual shape and suggests that there may be some exciton coupling between tryptophans that are close in space and in different relative locations than in the organic solvent structures (for which no apparent exciton interactions are seen). Similar results are obtained for a wide range of protein-to-lipid ratios from 1:15 to 1:363 (Fig. 3), if samples are examined under conditions that minimize scattering. This result is in contrast to that reported by Ovchinnikov and Ivanov (1983), who suggested the very different spectra they obtained at low peptide ratios corresponded to a completely different structure formed by association of three or more monomers. However, the spectra they reported appear to be dominated by lipid effects rather than caused by a different gramicidin conformation. In summary, our CD studies show that gramicidin adopts very different conformations in solution and in membranes and that, depending on the environment, both of the general folding motifs (helical dimer and double helix) originally proposed by Urry (1971) and Veatch et al. (1974) may be found.

A further demonstration of how the gramicidin folding motif varies with environment is seen in CD studies (Masotti et al., 1980) done in detergent solution. While the spectrum of gramicidin in these complexes resembles the spectrum of gramicidin in vesicles more closely than that in organic solvents, it is not identical to the spectrum of the phospholipid membrane structure. Furthermore, depending on the conditions used in sample preparation, the spectra seem to vary considerably in the lysolecithin detergent environment (Urry et al., 1982; Urry et al., 1983*a* and *b*), suggesting that a mixture of conformers may be present in different proportions in different experiments. Thus, gramicidin seems to be very sensitive to its environment. Even the presence of an amphipathic molecule in an ordered environment is not sufficient to cause it to adopt the membrane conformation.

To examine a functionally related feature of the molecular structure, we determined the effect of binding monova-

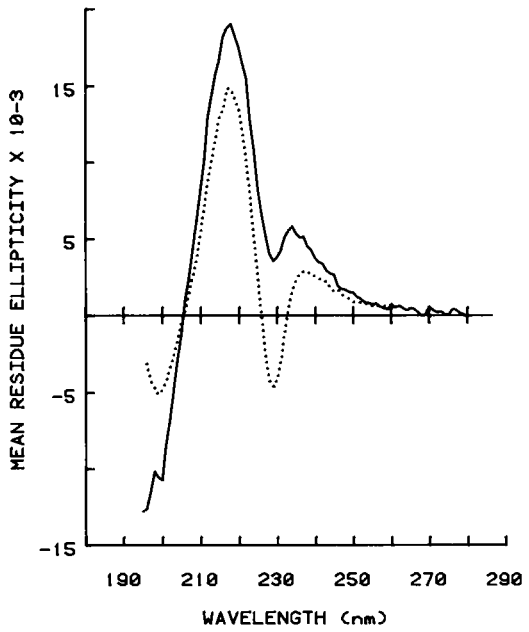

FIGURE 3 Circular dichroism spectra of gramicidin A in phospholipid vesicles at protein-to-lipid ratios of 1:30 (...) and 1:363 (—).

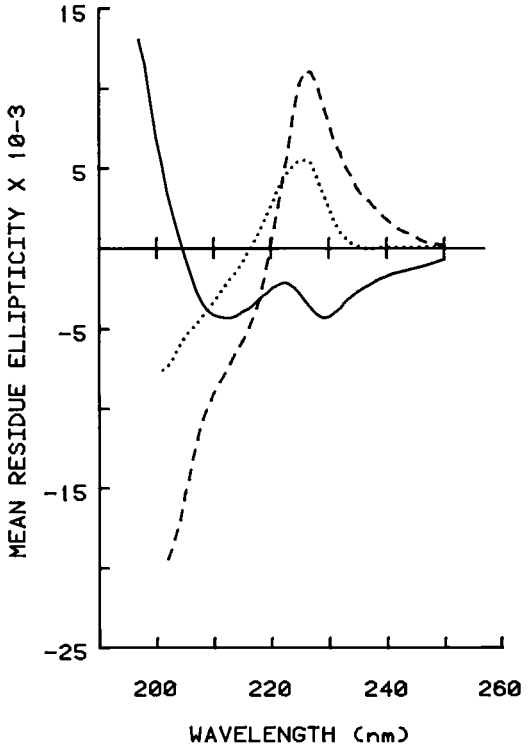

FIGURE 4 Circular dichroism spectra of gramicidin A in methanol in the absence of ions (—) and in the presence of saturating concentrations of Cs^+ (---) and Li^+(...).

lent cations to the molecule in either organic solvents or membranes (Wallace et al., 1981; Kimball and Wallace, 1982; Wallace, 1983). When the organic solvent form binds cations, a large change in its spectrum occurs (Fig. 4), corresponding to a major change in conformation of the molecule. Not only is the magnitude increased approximately twofold (as a consequence of a change in pitch of the helix), but also the sign of the curve (and hence the hand of the helix) is reversed when saturating amounts of cesium are bound (Kimball and Wallace, 1982). This spectrum is the same as that obtained for dissolved crystals of a gramicidin/cesium complex (data not shown). Furthermore, the decrease in pitch when cesium is bound, estimated from these CD data, corresponds well with the differences in helical repeat (Table I) and subcell dimensions of the molecule in crystals formed with and without cesium ions (Koeppe et al., 1978; Koeppe et al., 1979; Kimball and Wallace, 1984). The spectrum obtained using saturating amounts of the smaller ion lithium (Fig. 4) is also opposite in sign when compared with the spectrum for the polypeptide without ions, but is smaller in magnitude than that for the cesium form, indicating a larger pitch and suggesting that the pore is not as large for the lithium ion. The ion binding sites can be titrated to saturation (Fig. 5), and Scatchard analysis indicates that all the sites for each ion have similar binding constants, although the binding constants for lithium and cesium differ by approximately two orders of magnitude (Wallace, unpublished results). This suggests an interesting possible mechanism for ion binding in which, after binding ions, the molecule foreshortens and widens and, when it releases the ions, extends

TABLE I
GRAMICIDIN CRYSTALS

	Space Group	Unit Cell Dimensions	Helix Axis	Helix Repeat (Å)	Resolution (Å)
Gramicidin/Cs-I*	$C222_1$	33.9 × 35.9 × 91.4	c	5.1	2.9
Gramicidin/Cs-II*,‡	$P2_12_12_1$	32.1 × 52.1 × 31.2	b	5.2	1.5
Gramicidin-I§	$P2_1$	15.2 × 26.7 × 31.7	8° from c	5.4	1.3
Gramicidin-II§,‖	$P2_12_12_1$	24.8 × 32.4 × 32.7	c	5.6	1.0
Gramidicin/DMPC	$P2_12_12_1$ or $P2_12_12$	26.2 × 27.3 × 32.7	c	5.3	2.9

*Kimball and Wallace, 1984
‡Wallace and Hendrickson, 1985
§Koeppe et al., 1978
‖Wallace, 1983

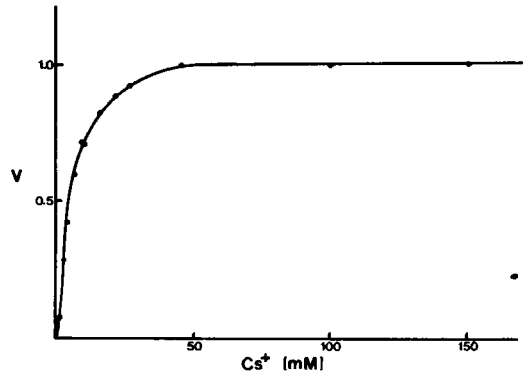

FIGURE 5 Binding curve (v, the reaction coordinate = gramicidin$_{bound}$/gramicidin$_{total}$, vs. ion concentration) for gramicidin A (7×10^{-4} M) and CsCl.

to the longer narrower pore. One could envision this as a channel gating mechanism. However, no such differences occur in the spectra of the membrane-bound form when it binds saturating amounts of ions (Fig. 6), suggesting that no change in pitch or in overall secondary structure occurs in the membrane-bound channels. This is reasonable because the native channel should be sufficiently large to accommodate even cesium ions. On the other hand, the organic solvent form may enlarge to accommodate the cesium because that pore must also accommodate solvent (methanol) or counterions (chloride) (Wallace and Hendrickson, 1985). These differential effects of ion binding are another demonstration that the conformations differ in the two environments (Wallace, 1983), and provide information on one functionally-related aspect of the structure.

Another functionally-related structural feature is the effect of membrane thickness. Two scenarios were envisioned for insertion of short (~30 Å) gramicidin molecules into thick (~45 Å) membranes: puckering in of the lipid molecules to accommodate the invariant gramicidin structure (Urry et al., 1975) or elongation of the gramicidin by refolding to accommodate the invariant lipid molecules. These possibilities could be distinguished by CD measurements. Puckering was expected to result in no difference between the spectra in thick and thin membranes, while

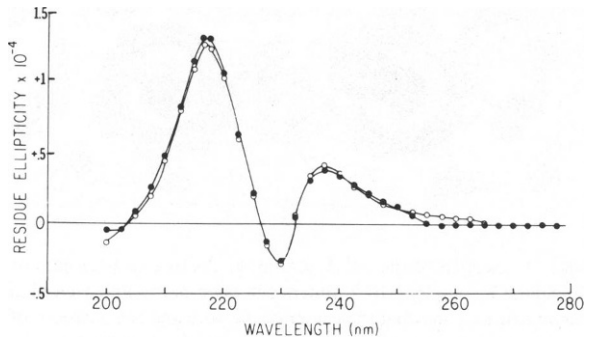

FIGURE 6 Circular dichroism spectra of gramicidin A in phospholipid vesicles in the presence (○) and absence (●) of 2 M CsCl (from Wallace et al., 1981).

elongation would result in a decrease in the magnitude of the 237 nm peak, corresponding to an increase in the helical pitch in the thicker membranes. Neither of these results was observed. Instead, a completely different spectrum was obtained, which corresponded to the spectrum of an inactive monomer, as confirmed by comparison with chemically modified molecules that do not form dimers (Wallace et al., 1981). This result suggested that the consequence of thicker membranes is dissociation of dimers so they no longer form conducting channels. This is in accord with conductance measurements (Kolb and Bamberg, 1977) indicating decreases in the mean channel lifetimes in thicker membranes. Hence, an observed functional property can be correlated directly with a structural feature of the channel.

These and other studies suggest that spectroscopy can provide useful information on structural features of the gramicidin molecule. Ultimately, however, one would like to have detailed molecular information on the polypeptide backbone, side chain, and ion binding site structures, which spectroscopic studies have not provided. The most suitable method for determining these features at high resolution is x-ray diffraction of well-ordered single crystals of the

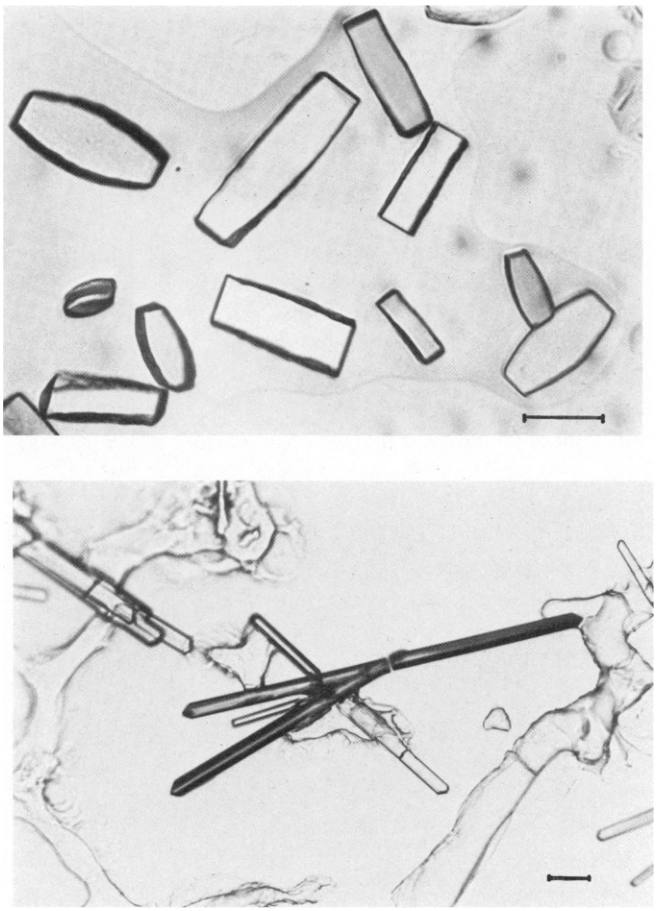

FIGURE 7 Photomicrographs of single cystals of gramicidin A currently under study: (*a*) gramicidin/Cs-II and (*b*) gramicidin/lipid complexes. The bars on each figure represent 0.02 mm. Unit cell parameters for these crystals are listed in Table I.

molecule. Crystals of gramicidin were first prepared 36 years ago (Hodgkin, 1949). However, until recently they remained intractable to solution; even the folding motif of the polypeptide backbone in the crystals had not been determined (Cowan and Hodgkin, 1953; Olesen and Szabo, 1959; Veatch, 1973; Veatch et al., 1974; Koeppe et al., 1978; Koeppe et al., 1979; Koeppe and Schoenborn, 1984). This may be because gramicidin falls in the difficult intermediate size range for crystallographic studies: too large for direct methods traditionally used on small molecules and too small to produce suitable heavy atom derivatives for multiple isomorphous replacement methods of macromolecular crystallography. Forming isomorphous heavy atom derivatives has been especially difficult because this molecule has only hydrophobic side-chains with limited capacities for binding heavy atoms and the crystals have relatively low solvent contents.

We have prepared new crystals (Fig. 7 and Table I) of a gramicidin/ion complex formed from cesium chloride (Kimball and Wallace, 1984) that diffract to 1.5 Å resolution (Fig. 8). This crystal form contains two gramicidin dimers and eight cesium sites per asymmetric unit. Because of the high cesium content, the Bijvoet differences from the cesiums were calculated to be ~18%, with a cesium partial structure contribution of ~80% in F. This suggested that single wavelength anomalous scattering might be used for phasing from a single native data set (Wallace and Hendrickson, 1984, 1985). The average measured anomalous differences were ~8% with a cesium partial structure contribution of 43%. The lower value for the measured anomalous differences relative to the calculated ones may be because of symmetries in the crystals which result in systematic absences and because of partial disorder in the cesium sites. The solvent volume in these crystals was calculated to be 24%. A 1.8 Å resolution map

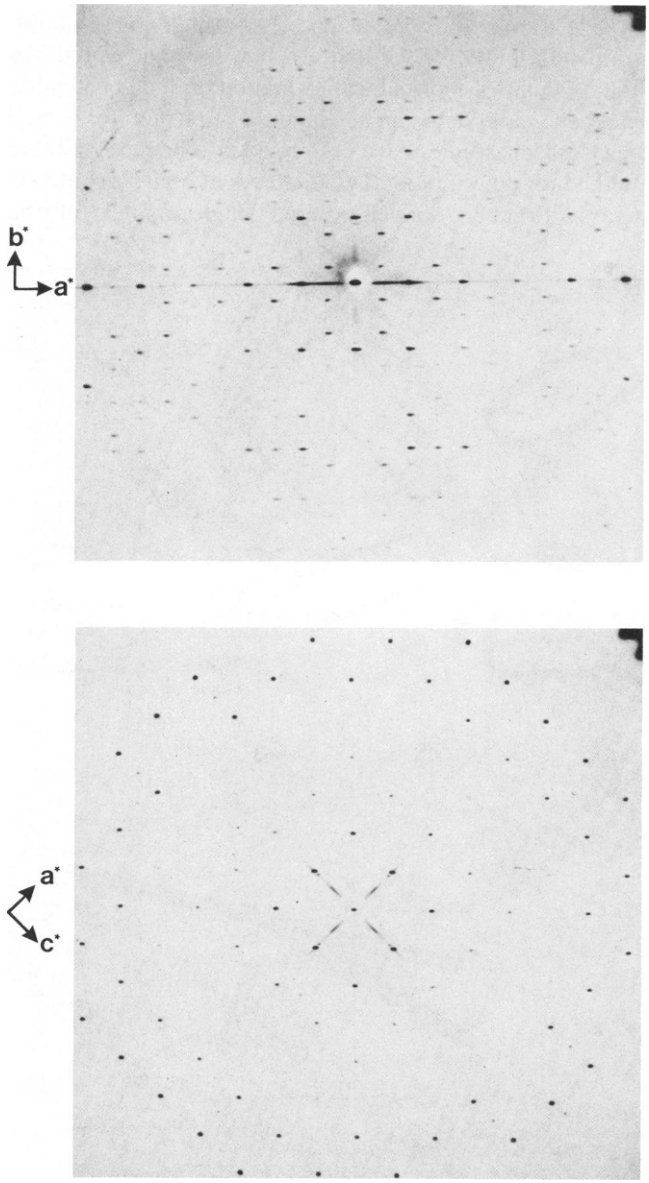

FIGURE 8 Zero layer diffraction patterns of gramicidin/Cs-II, crystals taken (*a*) parallel to b*, the helix axis, and a* (from Kimball and Wallace, 1984) and (*b*) parallel to a* and c*.

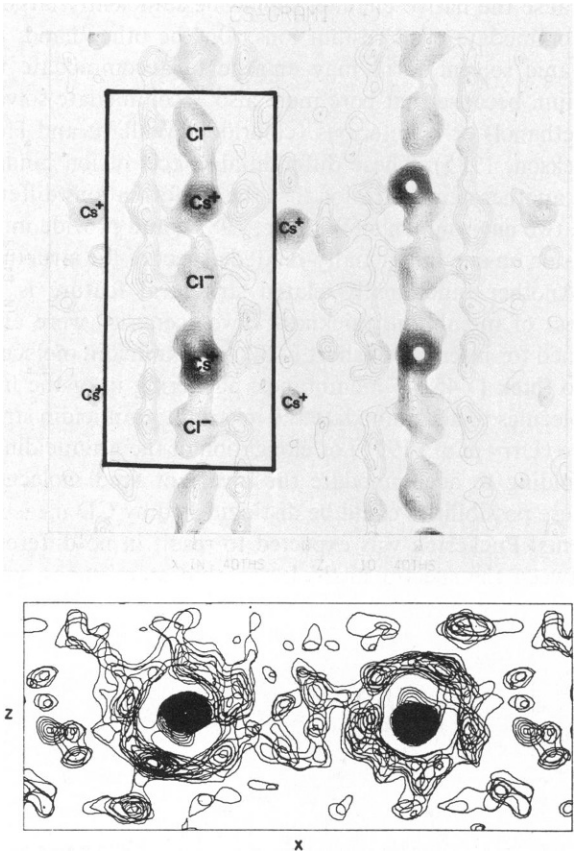

FIGURE 9 Section of the 1.8 Å resolution electron density map of the gramicidin/Cs complex. (*a*) 2 dimers per asymmetric unit viewed along the helix axis (*y*), showing the center of the pore and the locations of the cesium and presumed chloride ions. The approximate boundaries of the dimer backbone are indicated. (*b*) Section through the helix axis showing cesium ions in the pore and the axial arrangement of tryptophan side chains on the periphery of the pore.

has been calculated using these data (Fig. 9) and a molecular model has been fit to the preliminary electron density map, showing the folding of the backbone and positions of the side chains (Wallace and Hendrickson, 1984, 1985; Wallace and Hendrickson, unpublished results) (Fig. 10). Refinement is in progress.

The gramicidin/cesium crystals contain four independent monomers (i.e., two dimers) per asymmetric unit. All monomers appear to adopt similar backbone conformations, since the correlation coefficient between dimers is ~0.8 to a radius of 8 Å, and the correlation within a dimer is ~0.43.

The dominant feature of the dimers is a tubular structure with a central cavity containing the cesium ions, much as was inferred from the Patterson analysis of Koeppe et al. (1978). However, since the anomalous data permit phasing, Fourier maps could be calculated for these crystals, and at this resolution, the folding of the backbone and positions of the side chains could also be seen, thereby resolving the ambiguity as to molecular fold. The dimer appears to be a left-handed double helix formed from two antiparallel β-strands with six residues per turn and a helical repeat of 10.4 Å (Fig. 10). The types of hydrogen-bonding patterns involved are illustrated schematically in Fig. 11. The dimer is ~26 Å long with a central pore of ~5 Å diameter. Side chains are located axially on the perimeter of the pore (Figs. 9*a* and 9*b*), as a consequence of the alternating pattern of L- and D-amino acids in the sequence. In a typical α-sheet-type structure, side chains protrude alternately from the top and bottom sides of the sheet. However, with an alternating L- and D-sequence, all side chains protrude from the same side of the sheet. Hence, when the sheet is rolled up to form a helix, all side chains are on the periphery and the center of the helix forms a pore that can accommodate the ions. This results in very efficient use of polypeptide chain, since forming an equivalent size pore from α-helices (which have no such hole down their center) would use a bundle of ~6–7 helices, and would require five to six times as much polypeptide chain. The tryptophan rings do not appear to be stacked in parallel, consistent with the observation of the absence of

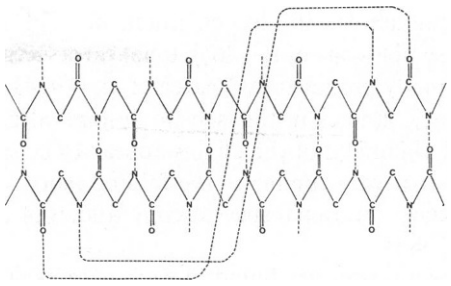

FIGURE 11 Schematic diagram showing hydrogen-bonding patterns for the molecular model of gramicidin/cesium complex.

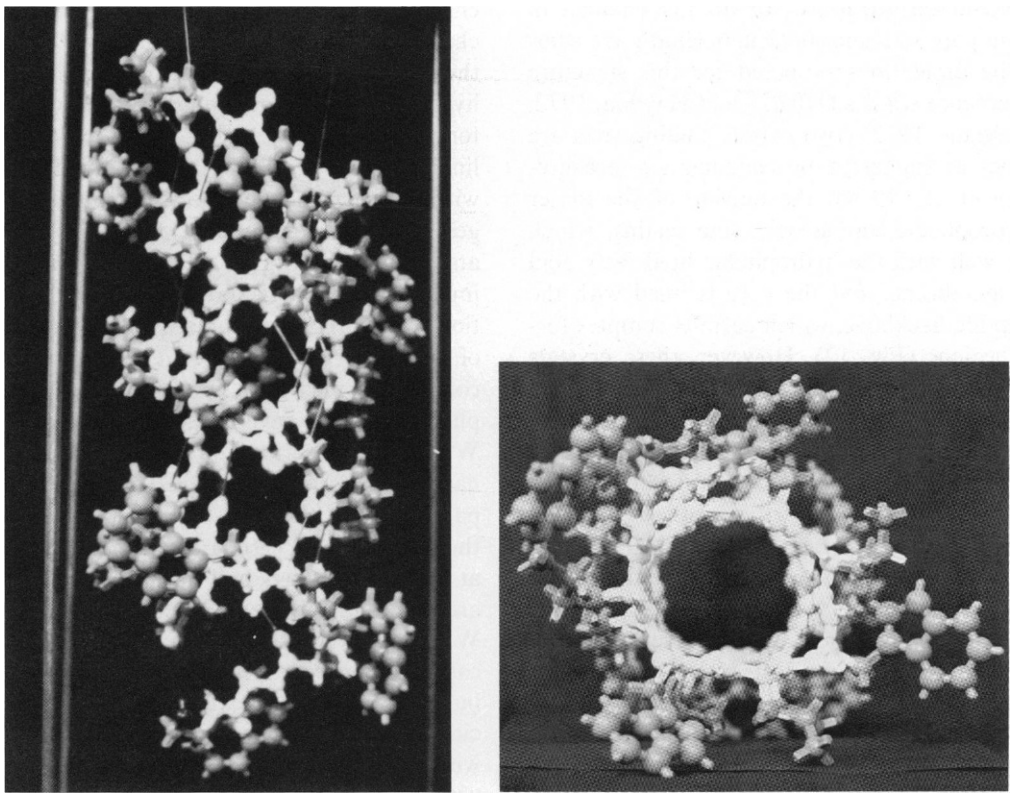

FIGURE 10 Preliminary molecular model of gramicidin/cesium built based on the 1.8 Å electron density map. (*a*) View along helix axis and (*b*) view down helix axis. Please refer to the color figure section at the back of this book.

exciton interactions in this environment. The structure closely resembles one of the double-helical structures (Fig. 1 *C*) originally proposed by Veatch et al. (1974) based on spectroscopic studies in this solvent system, although the pitch and alignment of the chains appear to be somewhat different. It is very similar to the folding motif suggested by the recent vibrational spectroscopy studies of Naik and Krimm (1984).

Two cesium sites are found in each pore located 7.1 Å from each end and separated by 11.8 Å; two partially occupied cesium sites are also found between dimers in the crystal (Fig. 9). There are also three strong features in each dimer that appear to be chloride ions.

While the zero layer diffraction patterns (Fig. 8) of this crystal form are virtually identical to those of a crystal form prepared from CsSCN in a different manner and which diffracts only to ~2.7 Å (Koeppe et al., 1979), we have found a different assignment for the space group and cesium sites based on Patterson maps (Wallace and Hendrickson, unpublished results; Wallace and Hendrickson, 1984, 1985). The similarity of the diffraction patterns suggests that although the other form has not been solved to high enough resolution to determine the backbone conformation, the molecular folds in the two crystal forms must be similar. The differences in space group and cesium sites, if real, may be a consequence of the different counterions present.

The gramicidin/cesium structure has a number of features which would be attractive for an ion channel in membranes: the pore size and molecular length are compatible with the dimensions proposed for this structure based on conductance studies (Hladky and Haydon, 1972; Myers and Haydon, 1972); two cation binding sites are found per dimer as suggested by conductance measurements (Hladky et al., 1979); the outside of the dimer consists of hydrophobic amino acid side chains, which would interact well with the hydrophobic lipid fatty acid chains in the membrane; and the pore is lined with the hydrophilic peptide backbone, which permits complex formation with the ions (Fig. 12). However, these crystals were formed from an organic solution of gramicidin, and a wide variety of spectroscopic (Wallace, 1983) and conductance (Finkelstein and Andersen, 1981) evidence indicates that the double helix structure is not consistent with the molecular fold in membranes. Furthermore, CD studies (Wallace, 1984) examining the process of gramicidin incorporation into membranes shows that while the double helical form may bind to vesicles, during integration into the membrane bilayer, the molecule is converted to the other (presumably helical dimer) structure. Hence, the gramicidin/cesium crystals provide the first detailed information on an intermediate in the process of insertion into membranes. In the future, it will be of interest to understand the dynamics and energetics of how the 28 intermolecular hydrogen bonds in the membrane-bound form of the molecule break and reform a new set of hydrogen bonds in the membrane-inserted form of the channel.

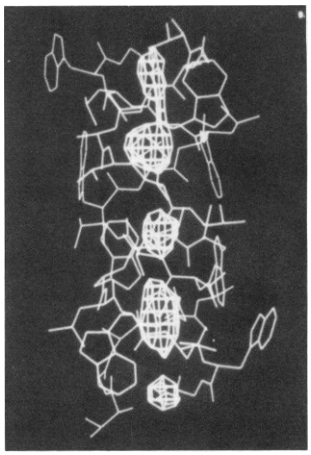

FIGURE 12 FRODO display of initial coordinates of the gramicidin/cesium structure. The two polypeptide chains are shown in different colors. The cage-like structures represent the densities of the cesium and chloride ions in the pore. Please refer to the color figure section at the back of this book.

It is also of interest to understand why this structure is not the structure of the active channel in membranes, and also to determine structural details of the channel conformation. Some progress has been made in both these areas. Specific features of the lipid molecules needed to induce channel formation have been investigated. The mere presence of lipid in the solution is not sufficient to induce channel formation (Wallace et al., 1981) suggesting that the ordered juxtaposition of hydrophilic head groups and hydrophobic tails is important. Studies using bilayers formed from lipid molecules in which the ester group linking the head group and fatty acid has been replaced with an ether linkage (Wallace, unpublished results) suggest that a specific interaction between the lipid carbonyl and carboxyl-terminus of the polypeptide may be an important feature in stabilizing the membrane conformation. To address the problem of the detailed conformation of the membrane-bound form of the molecule, crystals of a complex formed from gramicidin and dimyristoyl phosphatidylcholine have also been prepared (Wallace, 1983; Wallace, manuscript in preparation) (Fig. 7), and preliminary characterization (Table I) indicates that the unit cell parameters of this crystal form are different from any of the crystal forms without lipid. This finding suggests that at least the packing, and likely the molecular fold as well, are different. Raman spectroscopy (Wallace, 1983; Short, Wallace, Myers, Fodor, and Dunker, submitted for publication) comparing these crystals and gramicidin in vesicles indicates that the backbone conformation of the gramicidin in the lipid co-crystals is different than in the crystals without lipid, and that it is similar to the conformation in vesicles. Hence, there is reason to believe that solution of this crystal form will lead to an understanding of the structure of the molecule as found in membranes.

These studies point out the importance of using a variety of complementary techniques in the determination of functionally-relevant structures, especially for relatively small and flexible polypeptide molecules. Without the background of spectroscopic and conductance data, we might have believed that the structure of the molecule in the gramicidin/cesium crystals represented the membrane form of the molecule, as that structure corresponds well with most of our expectations for such a channel. Instead, the gramicidin/cesium crystals have given us a view of one of the intermediates in the insertion process.

In summary, using a combination of spectroscopic and diffraction techniques, it has been possible to examine the conformation and some functionally-related structural features of the gramicidin molecule. The conformation of gramicidin has been the subject of study in a number of laboratories for many years. A variety of different models has been proposed for its structure. CD spectroscopy has demonstrated that the molecule can adopt different conformations in different environments, but the actual structures in these environments were not known. X-ray crystallography has now permitted examination of the structure of a gramicidin/cesium complex at high resolution. This is the first crystal structure of any form of gramicidin to be solved. The double helical dimer in this crystal form corresponds to the pore present in organic solvents and is an intermediate in the membrane-insertion process. It shows the interactions of cesium and chloride ions with the the gramicidin polypeptide backbone. In the future, comparisons of this structure with the membrane-bound structure should provide a more complete understanding of the conformation, function, and folding of the gramicidin ion channel.

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DISCUSSIONS

Session Chairman: Donald L. D. Caspar
Scribes: Michael Roberts and Robert Bruccoleri

CASPAR: Your report illustrates an important point in structural biology. It often turns out that there is more than one structure, that the crystalline phase is often the one which is not biologically active.

RAGHAVENDRA: Your CD spectra in organic solvent showed a curve corresponding to α-helix even though the structure is antiparallel β-sheet.

WALLACE: If you look at Fig. 2 in our paper you will see that the spectrum of gramicidin is not that of an α-helix. An α-helix typically shows minima at 208 and 224 nm which are not present in these spectra. A regular β-sheet has amino acid side chains protruding on alternating sides of the sheet. Gramicidin has alternating L- and D-amino acids. Consequently, this sheet will have all its residues on one side. This enables it to be rolled into helical form with a hole down the middle. As a result, the ϕ and ψ angles differ from α-sheet-like structures.

RAGHAVENDRA: The putative exciton splitting seen in the CD spectra may be due to the presence of an α-helical like hydrogen bonding structure, but substantial differences in the ϕ and ψ torsion angles from an α-helix. Second, there are four tryptophan residues in gramicidin A which could influence that region of the CD spectra.

WALLACE: We believe the exciton splitting is due to the tryptophan transitions only, not the backbone. About 1/3 of the molecule is tryptophan and there is an aromatic $\pi \rightarrow \pi^*$ transition in this region which is substantially greater in magnitude than the $n \rightarrow \pi^*$ transition of the peptide backbone.

RAGHAVENDRA: Is the sample of gramicidin A in membrane a solution or a suspension?

WALLACE: It is a suspension of very small unimolecular vesicles, ~ 250 A in diameter.

RAGHAVENDRA: Would this cause scattering artifacts in the CD spectra?

WALLACE: Differential scattering properties are important to consider when examining particles that are large relative to the wavelength of the incident light (see Wallace and Mao. 1984. *Anal. Biochem.*. 142:317–328). We found no differential scattering for these small particles, as demonstrated by varying the acceptance angle used in the CD measurements.

CORNELL: Given the fact that the channel is made up of the backbone of the molecule, with all the side chains directed away from it, what are your speculations as to why the channel conductance is dependent on the amino acid side chains?

WALLACE: There are two features that are affected by the amino acid side chains: the lifetime and conductance of the channel. Olaf Andersen and his colleagues have done a number of systematic studies on this.

DURKIN: Electrostatics is one factor. E. Barrett in Olaf Andersen's lab made several systematic series of position 1 substitutions in which the polarity of the amino acid increased while the side chain remained essentially isosteric. For example, we substituted trifluoro- and hexafluorovaline for valine. The finding is that the conductance decreases as the side chain polarity increases. This is exactly what you would predict for an ion-dipole interaction in that position.

CORNELL: Do the electrostatic factors influence the electrostatic field down the interior of the pore, or in some way modify the structure of the gramicidin when the ion is passing down the center?

DURKIN: I had been thinking in terms of the first of those possibilities, and the second had not occured to me.

BAYLEY: Can you see a conformational route that would enable the transformation between the end-to-end and double helical structures?

WALLACE: We have looked at the process of binding this molecule to membranes, and it may be this double helical structure is an intermediate in the insertion process. We find that the CD spectrum of the molecule when it initially binds to the membrane corresponds to that of the double helical structure. At that point there are few, if any active conducting channels. The structure conversion would involve the breaking and remaking of 28 hydrogen bonds, and I would envision it occurring in individual steps of unwinding rather than one concerted step.

BAYLEY: Since you can study this spectroscopically, does that thermal process look highly cooperative?

WALLACE: We have not done studies of fine enough resolution to detect any other intermediate-type structures.

BAYLEY: It looks as though one form of helical structure could be extruded from the other with an almost complete conservation of the number of hydrogen bonds, so you would not necessarily need such a large activation energy.

GLAZER: How soluble is gramicidin in water?

WALLACE: It is virtually insoluble in water. The partition coefficient into a lipid bilayer is very high. In the vesicle preparations we use, no polypeptide can be detected in the aqueous phase.

GLAZER: Then how do you visualize the parallel alignment with the polar surface?

WALLACE: We do not have data now that would distinguish between a parallel or a perpendicular orientation of the initially bound (not membrane-inserted channel) form.

DREXEL: Some type of cross-linking would be a way to establish whether the molecules would be attached end-to-end or in a double helix.

WALLACE: Conductance studies on NH_2-terminal cross-linked molecules have been done by Urry et al. (1971. *Proc. Natl. Acad. Sci. USA.* 68:672) and Bamberg et al. (1977. *Biophys. Biochim. Acta.* 465:486) and their colleagues. These studies also indicate the existence of the end-to-end dimer with the N_2-termini located in the middle of the membrane. Ovchinnikov and Ivanov (1983. *In* Conformation in Biology, Seinwasan and Sarma, editors., 155–174.) has recently cross-linked the NH^2-terminus of gramicidin to the COOH-terminus of another gramicidin molecule which could form a double helix but not helical dimer structure, and finds conducting channels for this case. In fact, our NMR experiments could not exclude up to 10% of the double helical form as a conducting molecule.

DREXEL: Has anyone tried to crystallize the cross-linked species?

WALLACE: We have considered that might be a way to force the crystallization of the *N–N* helical form, but since we now have good lipid/gramicidin co-crystals which appear to have the molecule in the channel conformation, the use of the cross-linked dimer will be unnecessary.

KAPLAN: Some years ago, we synthesized a compound, NH_2-palmitoyl-serine, which was designed to influence the hydrogen bond network of water to enhance the solubility of lipids. It worked for us, and it may permit you to study gramicidin in free aqueous solution without vesicles, and it may help you understand how the channel is working.

WALLACE: Gramicidin is very sensitive to its environment, and it adopts a different conformation depending on the solvent and amphipathic molecules present. In lysolecithin (a detergent), it forms a different conformation than in membranes. We've also examined gramicidin in gangliosides; in that environment, it also does not adopt a membrane conformation.

KAPLAN: We didn't use N-palmitoyl serine in the ionic detergent form; we kept it carboxyl, not carboxylate. It's not acting like a soap; hydrogen bonds to itself and to the surrounding aqueous medium (Kaplan. 1967. *J. Colloid and Interface Sci.* 25:63–70).

McPHERSON: What is the current state of refinement for this structure, and what precautions did you take to optimize the measurement of the anomalous dispersion?

WALLACE: Elimination of systematic differences is important in data collection because these expected anomalous differences are not very large. Calculated anomalous differences were 18%, whereas measured values were only 8%, which meant we had to be very careful in collecting pairs of data. We collected sets of 20 reflections and then their Freidel mates, so measurements were made close in time to minimize systematic errors due to radiation damage. Our refinement is in an early stage and currently at R = 35% at 1.8 Å resolution. The molecule is difficult to refine because the cesiums contribute tremendously to the scattering. A small change in their position changes the R-factor substantially.

BLANK: First, your talk does not illustrate convergent techniques, but rather divergent results. This leads to a question about what each technique means and which one to trust regarding the situation in the membrane. Second, instead of saying "physiologically best characterized," you really mean "physically best characterized." These are not physiological systems.

WALLACE: No, by that we mean that it has the best-characterized conductance properties of any ion channel.

BLANK: How does gramicidin tell us something about physiological systems? In most pores, a number of helices form the pore with the helix exteriors forming the channel wall.

WALLACE: Gramicidin is a unique structure because of its alternating D and L-amino acids. The knowledge of structure of the ions, water, and functional groups in the channel may have more general application. We chose a system where we could relate the high resolution structure to the conductance properties. Now, we are also working on crystallizing other, more complex, channels.

CASPAR: Not all channels are α-helices and what you have here is β-sheet structure. Porin is presumably a β barrel with the strands running normal to the plane of the membrane, but the gap junction has strands running parallel to the plane.

One of your paper's referees points out that the CD spectrum indicates an exciton splitting between tryptophans and that different orientations of the tryptophans may be stable on time scales long enough to influence the conductance and are the tips stacked. Do you have a comment? Another referee asks, how mobile were the sidechains?

WALLACE: The CD measurements are of time-averaged structures so we can't say anything about the fluctuations. Alan Kleinfield's group (Boni et al., this volume) has looked at tryptophan fluoresence and has reported rapid reorientations. In the crystal structure, we do not see any stacking interactions between the tryptophans. This correlates with the absence of exciton splitting in the CD spectrin in organic solvents. But we do see the exciton peaks in membranes, which suggest stacking occurs in membrane. We should be able to say something about sidechain mobility from the temperature factors once we've completed the crystallographic refinement.

DURKIN: We know very little about membrane protein pores. There are many models, but hard structural data give low-resolution blobs, and we don't know what secondary structure elements they contain. Also, Stephen Kennedy (Kennedy et al. 1977. *Science (U.S.A.)* 196:1341–1342) synthesized β-helices of all L-amino acids. In this case, the sidechains pointed into the pore. This demonstrates that there are a range of possible channel structures.

Why do we think the anti-parallel double helix is not the channel structure? It should be as good as head-to-head single helices. All the evidence is that the membrane conformation is the head-to-head dimer, but this evidence all deals with the average properties of a large number of channels. In Olaf Anderson's lab we measured single channel conductances. Histograms of current transitions (Durkin et al., this volume) have a main peak representing 85–95% of the measurements, but there are other lower current events. Even the original work of Hladky and Haydon demonstrated current events of nonstandard size. Some of these may be chemical impurities, others oxidized tryptophans, others mini-conductance events. In the presence of pure valine gramicidin A, we see single-channel events about one-tenth of the time that are very long lived, lasting minutes. Other gramicidin workers I've talked to also see these events. We speculate that these are channels formed by anti-parallel intertwined dimers as found in Bonnie Wallace's crystals. One would expect that the large number of hydrogen bonds would stabilize the structures for a long time. We really need to discover conditions that would favor the formation of intertwined structures.

CASPAR: Another referee asks why gramicidin does not transport chloride.

WALLACE: That may be an overstatement. George Eisenman (1977. *In* Metal-Ligand Interactions in Organic Chemistry and Biochemistry. Pullman and Goldberg, editors. 1–26) has found some chloride permeability in the presence of thallium, and also that anion species may influence cation conductance. In our crystals, which were grown at high ionic strength, there may be some competition of ions for water sites, so whether the chlorides are in the pore in solution at lower location concentrations is not clear.

CORNELL: I fully appreciate Martin Blank's comments on the pseudo-protein status of gramicidin. Nonetheless, it is important to study this simple gramicidin system in order to gain insights into the dynamics of proteins in membranes. Ross Smith and I have synthesized a series of gramicidin molecules in which we have C-13 carbonyls labeled in three separate sites. Our results do not adjudicate whether we have a single helix or a double helix, but we have a structure that is consistent with a helix. Our membranes were lipid bilayers aligned on glass slides. In fluid bilayers, we see a cylinder reorienting about its long axis. If we freeze the lipid bilayer, the rotation stops. What we found to be particularly intriguing is that we can go $\sim 10°C$ below the lipid phase transition and still observe this reorientation. This may be associated with the P_{β}' phase transition which prevails in the DMPC we used. The NMR indicated the membrane was ordered, but the gramicidin was still rotating on the millisecond time scale. We had to go down to 280°K in order to stop the gramicidin rotation. By labeling the α-carbon and looking at its dipolar interaction with the carbonyl carbon, we could determine any wobble associated with the rotation. The off-axis wobble was $\sim 20°$. We have some vague results that do not indicate much tryptophan mobility.