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The Conformation of Gramicidin A†

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ABSTRACT: Gramicidin A is thought to form a dimer channel through which alkali cations and hydrogen ions can passively permeate lipid bilayer membranes. The present work describes four conformational species which have been isolated from a single organic solvent system and individually characterized by circular dichroism, proton nuclear magnetic resonance, and infrared spectroscopy. Three distinct conformations were found: two are probably helices of opposite handedness with predominantly parallel-β hydrogen bonding, and the other has predominantly antiparallel-β hydrogen bonding. All four of the conformational species may be dimers. To account for these observations, a new family of double helices is postulated.

Gramicidin A is a linear polypeptide with the following sequence (Sarges and Witkop, 1965): formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-(L-Trp-D-Leu)$_3$-L-Trp-ethanolamide. If glycine is thought of as a potential D residue, then the sequence is a strictly alternating LDLD; all side chains are relatively hydrophobic.

Gramicidin A facilitates the passive diffusion of the alkali cations and hydrogen ion through natural (Harold and Baarda, 1967; Chappell and Crofts, 1965; Harris and Pressman, 1967) and artificial lipid bilayer membranes (Mueller and Rudin, 1967; Myers and Haydon, 1972). There is strong evidence that gramicidin A forms a channel spanning the membrane hydrocarbon (Hladky and Haydon, 1972; Krasne *et al.*, 1971), and some evidence that the channel requires two molecules of gramicidin A (Tosteson *et al.*, 1968; Bamberg and Läuger, 1973).

In search of evidence to critically assess models for the conformation of the gramicidin A involved in the transmembrane channel, several groups of workers (Glickson *et al.*, 1972; Urry *et al.*, 1972; Isbell *et al.*, 1972; Rothschild and Stanley, 1974) have examined the conformation and aggregation of gramicidin in solution. Although a nonpolar organic solvent may be a good model environment for the membrane interior, it must be borne in mind that a lipid bilayer membrane is essentially a heterogeneous apposition of polar and nonpolar regions.

In the present work four conformational species have been physically isolated from a single nonpolar solvent system and individually characterized by circular dichroism (CD), infrared spectroscopy, proton nuclear magnetic resonance (^{1}H nmr) spectroscopy, and X-ray diffraction. Three distinct conformations were found: two are probably helices of opposite handedness with largely parallel-β hydrogen bonding; another has largely antiparallel-β hydrogen bonding. All four of the conformational species appear to be dimers. To account for some of these and other observations, a new family of parallel-β and antiparallel-β double helices is postulated. The structure of the gramicidin transmembrane channel is discussed in terms of the solution structures in the following paper (Veatch and Blout, 1974).

Experimental Section

Materials. The gramicidin used was a gift from S. B. Penick and Company (New York, N. Y.), once-crystallized from ethanol (85% gramicidin A, 10% gramicidin B, 5% gramicidin C, found by amino acid analysis). Gramicidin A was a gift from Dr. Erhard Gross of The National Institutes of Health. Desformylgramicidin and *N*-acetyldesformylgramicidin were prepared from gramicidin according to the procedure of Sarges and Witkop (1965). Methanol, ethyl acetate, and dioxane were Spectrograde (Matheson Coleman and Bell). Ethanol was "Rossville Gold Shield" (Commercial Solvents Corporation); dimethyl sulfoxide was "spectroanalyzed" (Fisher); and the chloroform was analytical grade (Baker).

Thin-Layer Chromatography. All silica thin-layer chromatography (tlc) plates were from Quantum Industries. The plates were developed in dioxane–water (100:1) (v/v) and visualized with the tryptophan reagent, *N,N*-dimethylaminobenzaldehyde (Greenstein and Winitz, 1961). Absolute spot mobilities relative to the solvent front were approximately 0.4 for species 4, 0.3 for 3, 0.15 for 2, and 0.04

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for 1. A spot of weak intensity at the origin did not interconvert and was not examined further; it is probably due to polar non-gramicidin impurities.

Preparation of Gramicidin Isolated Species 1, 2, and 4. Approximately 10 mg of once-crystallized gramicidin in methanol was banded along the bottom of a preparative tlc plate with 1 mm layer thickness and fluorescent indicator (*PQ1F-1000*). The plate was developed in dioxane–water (100:1). Immediately after the plate was removed from the developing tank, it was covered with a plastic sheet to prevent evaporation. The band positions were visualized under an ultraviolet lamp and marked. The band of interest was scraped, still wet, into a tube containing an aliquot of the eluting solvent. If more than one band per plate was to be scraped, then the remaining bands were covered with a glass sheet.

For species 4 the eluting solvent used was ethyl acetate–ethanol (4:1). For the "minimum interconversion" protocol, a small volume of eluting solvent was used, and the eluent was chromatographed directly on a Sephadex LH-20 column (2 × 30 cm) equilibrated with ethyl acetate to remove small molecular weight contaminants. The fraction with maximum absorbance (monitored at 290 nm) could then be dried down with a gentle stream of dry nitrogen with little species interconversion.

For the "high yield" protocol for species 4 (~10% yield), larger elution volumes were used; however, the eluent had to be concentrated prior to Sephadex LH-20 chromatography. The eluent solution was placed on a rotary evaporator, evaporated to about one-half its initial volume at room temperature, then pure ethyl acetate was added to restore the volume to its initial volume. After several such cycles, the ethanol concentration had been lowered sufficiently so that the solution could be reduced to the desired volume with only modest interconversion.

For the "minimum interconversion" protocol, species 1 and 2 were prepared separately using an elution solvent of dioxane–water (100:2) which was immediately diluted into a large excess of pure dioxane. The solution was successively partially evaporated and replenished with fresh dioxane, similar to species 4 above. After the water was removed, the solution was evaporated, ethyl acetate was added, and the solution was chromatographed as described for species 4. The "high yield" protocol used larger elution volumes and more evaporation at the expense of some interconversion.

For ^{1}H nmr work the solution from which the plate was banded was in methanol-d_4 (Thompson Packard), and the development was done with dioxane–D_2O (100:1). For species 1 and 2, D_2O also replaced H_2O in the elution. For species 4, CH_3CH_2OD–ethyl acetate (1:4) was used to elute.

Preparation of Species 3. Solutions of greater than 90% conformational purity were obtained by dissolving vacuum-dried crystals of gramicidin mixture slowly crystallized from ethanol or methanol. For ^{1}H nmr experiments the crystals were crystallized from CH_3OD or CH_3CH_2OD.

Circular Dichroism. All spectra were recorded with a Cary 60 with Model 6001 circular dichroism attachment. Concentrations were determined on a Cary 15 spectrometer from the absorbance at 290 nm, assuming an extinction coefficient of 20,000 cm^{-1} M^{-1}. Residue ellipticity was obtained by dividing the molar ellipticity (deg cm^2 $dmol^{-1}$) by 15. Cells with 0.10-mm path length were used with 0.050 ml of solution. All samples had optical densities of less than 2 in the region of interest, and the dynode voltage never exceeded 600 V. The sample temperature was between 20 and 25°. All of the magnitudes were reproducible to within 15%, provided the tlc assay showed greater than 90% of the material in the desired spot.

Infrared. A Perkin Elmer 521 infrared spectrophotometer with expanded wave number scale was used with matched 0.2-mm NaCl cells. The sample concentration was approximately 4 mg/ml and the sample temperature ~30°. The contribution of solvent to the spectra has been subtracted.

Proton Nuclear Magnetic Resonance. The spectra were recorded using a Varian XL-100-15 nmr spectrometer with a Varian 620/16K computer operating in Fourier transform mode. The chemical shifts are relative to tetramethylsilane (Me_4Si). All spectra were recorded at 1000-Hz sweep width utilizing 2048 computer digitization points. The pulse width was 25 μsec, acquisition time was 1.0 sec, and 500–1000 transients were collected. The dioxane-d_8 was 99% D (Stohler Isotope Chemicals). All chemical shift values are ±0.1 ppm.

Results

Thin-Layer Chromatography. One-dimensional silica thin-layer chromatography of gramicidin (or gramicidin A) shows four spots which will be denoted 1, 2, 3, and 4 in order of increasing mobility. A chromatogram, developed symmetrically in two dimensions with the same solvent, yields four spots along the diagonal (Figure 1a); however, if ethanol is used to interconvert the species on the plate prior to development in the second direction, a 4 × 4 grid of 16 spots results (see Figure 1b).

One-dimensional tlc was used to visually estimate the species composition of concentrated solutions of gramicidin in dioxane or ethyl acetate. Dried crystals of gramicidin (from ethanol or methanol), dissolved in dioxane and chromatographed, yielded species 3 alone; consequently, these dried crystals were used as a convenient source of this species.

Preparative one-dimensional tlc was used to prepare species 1, 2, and 4 as described in the Experimental Section. It was important to use the visual tlc assay to monitor the results. A "minimum interconversion" protocol was considered successful if >90% of the material was in the desired species (true for species 3 from crystals); the "high yield" protocol was considered successful if >70% was in the desired spot. Gramicidin (a once-crystallized mixture of 85% gramicidin A, 10% gramicidin B, and 5% gramicidin C) was used to prepare the species instead of the more scarce purified gramicidin A. In the following paper (Veatch and Blout, 1974), it is demonstrated that gramicidins B and C behave similarly to gramicidin A.

Circular Dichroism. The circular dichroism spectra of the gramicidin isolated species in dioxane are shown in Figure 2 (200–250 nm) and Figure 3 (250–320 nm). The spectra could not be recorded below 200 nm due to solvent absorption. In Figure 4 the spectra of the same samples, dissolved in 2-propanol, are given; species 1 and 2 interconvert rapidly in this solvent and, hence, only their sum is shown.

In Figure 3 the bands at 286 and 293 nm for species 1, 2, and 4 are assigned to the ^1L_b electronic transitions of indole, and the very broad band near 270 nm for all of the species is assigned to the envelope of the ^1L_a transitions (Strickland *et al.*, 1970). Similar bands are observed for species prepared from purified gramicidin A.

Species 1 and 2 have nearly identical CD spectra. Note

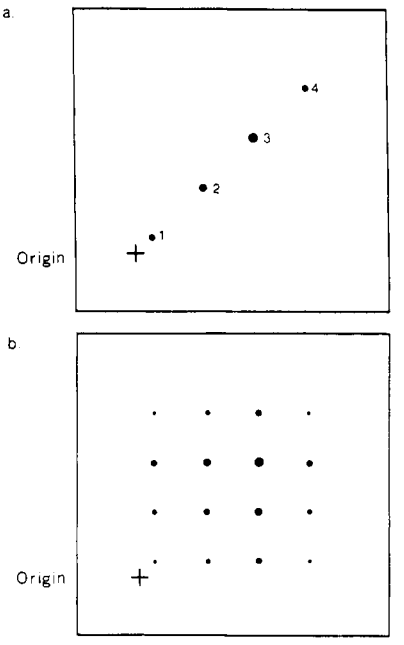

FIGURE 1: Two-dimensional thin-layer chromatography of gramicidin. In these schematic diagrams the dot size is proportional to the intensity of the spot. The plates were developed symmetrically in both directions with intermediate air drying: (a) without interconversion; (b) with interconversion.

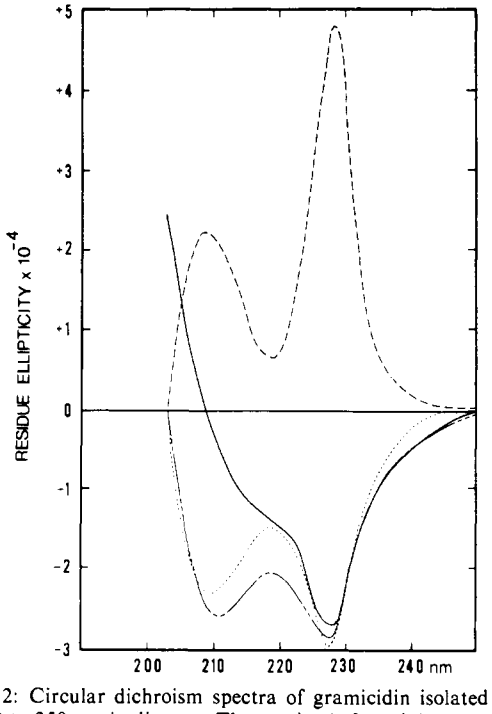

FIGURE 2: Circular dichroism spectra of gramicidin isolated species from 200 to 250 nm in dioxane. The species 1, 2, and 4 were prepared using the minimum interconversion protocols described in the Experimental Section. Species 3 was obtained from crystals. (---) 1; (-·--) 2; (—) 3; (— —) 4.

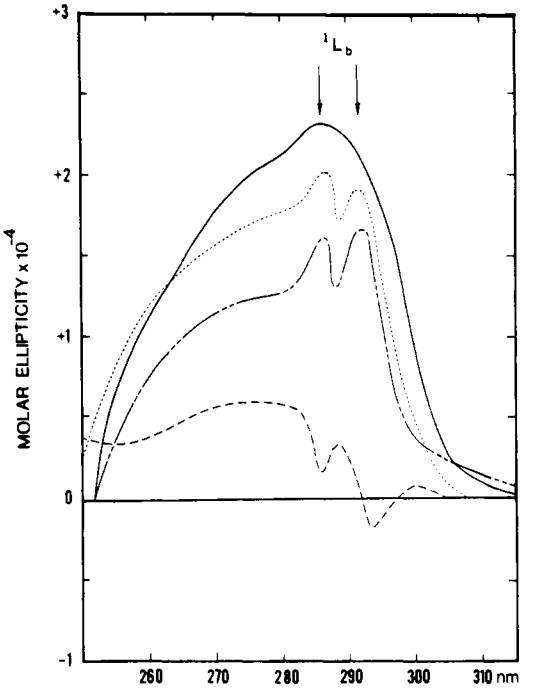

FIGURE 3: Circular dichroism spectra of gramicidin isolated species from 250 to 310 nm in dioxane. The samples for species 1, 2, and 4 were prepared using the high yield protocols described in the Experimental Section. Species 3 was obtained from crystals. (---) 1; (-·--) 2; (—) 3; (— —) 4.

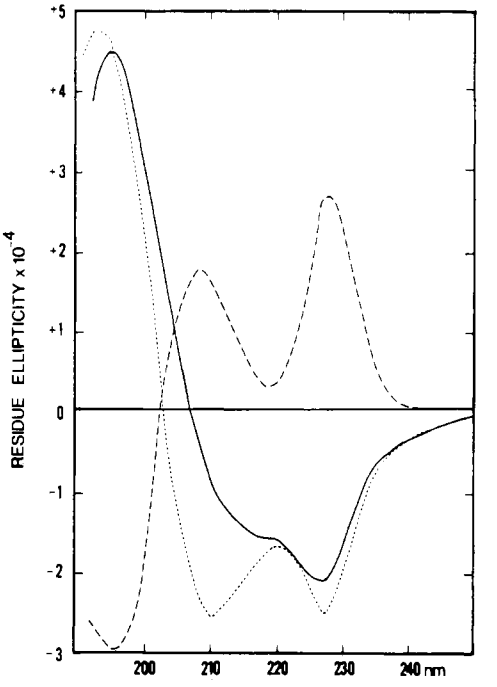

FIGURE 4: Circular dichroism spectra of gramicidin isolated species from 190 to 250 nm in 2-propanol. The samples used for Figure 2 were evaporated under dry nitrogen, dissolved in 2-propanol, and the spectra recorded within an hour. (---) 1 + 2; (—) 3; (— —) 4.

that the spectrum of species 4 is approximately the mirror image of that of species 1 and 2; with the exception of the aromatic ^1L_a band, all of the bands for species 4 are opposite in sign and comparable in magnitude with those of species 1 and 2. Species 3 has a spectrum which differs significantly from that of species 1 and 2.

Infrared. The infrared spectra of the gramicidin isolated species in the amide I and II regions are shown in Figure 5. Due to the similarity of species 1 and 2, only the spectrum of species 2 is shown. All of the species have a strong amide I band at 1633 cm^{-1}. Species 3 has, in addition, a resolved 1680 cm^{-1} amide I band. All species have broad amide II bands near 1545 cm^{-1}. The spectra in the NH region (not shown) have peaks at 3280 cm^{-1} for species 3 and at 3270 cm^{-1} for species 1, 2, and 4. The presence of the 1680-cm^{-1}

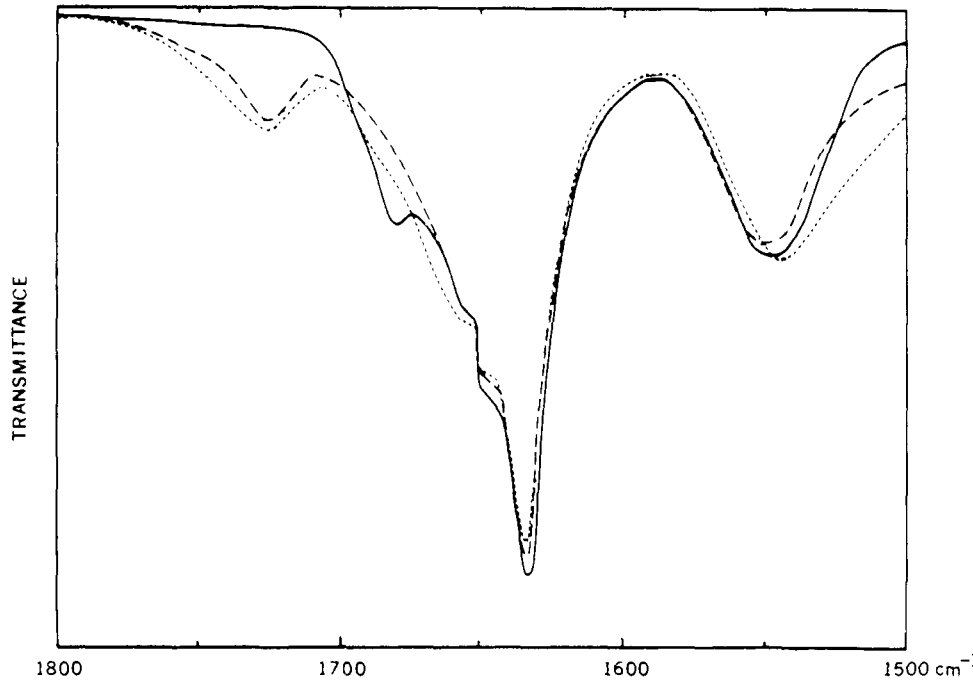

FIGURE 5: Infrared spectra in amide I and II regions of gramicidin isolated species in chloroform. Samples were prepared as in Figure 3. The peak at 1730 cm^{-1} is probably due to the carbonyl of ethyl acetate, which was used for the Sephadex LH-20 chromatography of species 1, 2, and 4. The spectrum of species 1 (not shown) is very similar to that of species 2. (---) 2; (—) 3; (— —) 4.

band for species 3 and its absence for the other species strongly suggest that it arises from conformational differences among the species. Species 3, prepared by tlc from *N*-acetyldesformylgramicidin, also shows the weak 1680-cm^{-1} band, which shows that this band is not due to the N-formyl carbonyl; however, the contribution of non-hydrogen-bonded carbonyls at the ends of the structure to the 1680-cm^{-1} peak is difficult to rigorously exclude.

Proton Nuclear Magnetic Resonance. The proton nuclear magnetic resonance spectra (100 MHz) of the gramicidin isolated species in dioxane-d_8 are shown in Figure 6. The species were partially deuterated. An expanded downfield region of this spectra is shown in Figure 7.

The peaks between 8.0 and 8.2 ppm in Figure 7 are assigned to the resonances of the formyl protons. The formyl proton of dimethylformamide is at 8.1 ppm in dioxane-d_8, and partially deuterated desformylgramicidin and *N*-acetyldesformylgramicidin lack peaks in this region. The integrated peak area between 8.0 and 8.2 ppm (normalized to 20 protons for the total indole protons between 6.5 and 8.0 ppm) is about unity for each of the species. The peptide NH's that would normally also occur in this region have been deuterated. In some preparations of species 2 the formyl resonance appeared to be symmetrically doubled, while in others it merely appeared to be broadened.

The peaks from 9.0 to 9.5 ppm are assigned to the indole NH's; although originally deuterated, they have evidently partially back-exchanged for protons from water in the solvent. The indole NH of *N*-acetyltryptophanamide occurs at 9.5 ppm in dioxane-d_8. Species 1, 2, and 4 have very similar indole NH resonances (9.4 and 9.5 ppm); and species 3 has a major peak at 9.4 ppm and a smaller peak at 9.0 ppm.

There are significant differences among the isolated species in the region above 1 ppm where the methyl resonances are found in DMSO-d_6 (Glickson *et al.*, 1972); in DMSO-d_6 the methyl protons of the valine and leucines adjacent to tryptophan in the sequence are shifted upfield by about 0.3 ppm relative to the other methyl protons of the leucine and valines. For species 3 three resolved peaks occur at 0.7, 0.3, and 0.1 ppm. If the 0.7-ppm peak is assigned to the unshifted methyls, then the other two peaks are shifted upfield by 0.4 and 0.6 ppm. None of the other species have the small peak at 0.1 ppm, and species 4 lacks the resolved peak near 0.3 ppm present for species 1 and 2.

Discussion

Sarges and Witkop (1965) reported multiple spots on thin-layer chromatography for chemically homogeneous valine-gramicidin A. The 4 × 4 grid reported here for gramicidin upon symmetrical two-dimensional tlc implies that the four gramicidin species are *interconvertible*. The kinetic stability of these species in nonpolar solvents is discussed in detail in the following paper (Veatch and Blout, 1974).

Data in the literature on the conformation of gramicidin in solution are not directly applicable to any one of the gramicidin isolated species. All data in nonpolar solvents have been recorded on a mixture of aggregated conformational species not at equilibrium (Isbell *et al.*, 1973; Rothschild and Stanley, 1974) or in relatively polar solvents, such as dimethyl sulfoxide (Urry *et al.*, 1972; Glickson *et al.*, 1972), where gramicidin is largely monomer (see the following paper, Veatch and Blout, 1974). Urry *et al.* (1972) did observe multiple formyl proton peaks in the ^{1}H nmr spectrum of gramicidin in methanol-d_4 which probably correspond to the different chemical shifts of the gramicidin isolated species.

The conformational properties which differentiate the gramicidin isolated species are summarized in Table I. Species 1 and 2 are very similar in all respects with the possible exception of the formyl proton resonance. Species 4 has a CD spectrum which is approximately the mirror image of that of species 1 and 2. In addition, species 1, 2, and 4 have very similar fluorescence and infrared parameters. Species 3 differs in many respects from species 1, 2, and 4, especially in the presence of the 1680-cm^{-1} infrared amide I band. It is likely that the structures of species 1, 2,

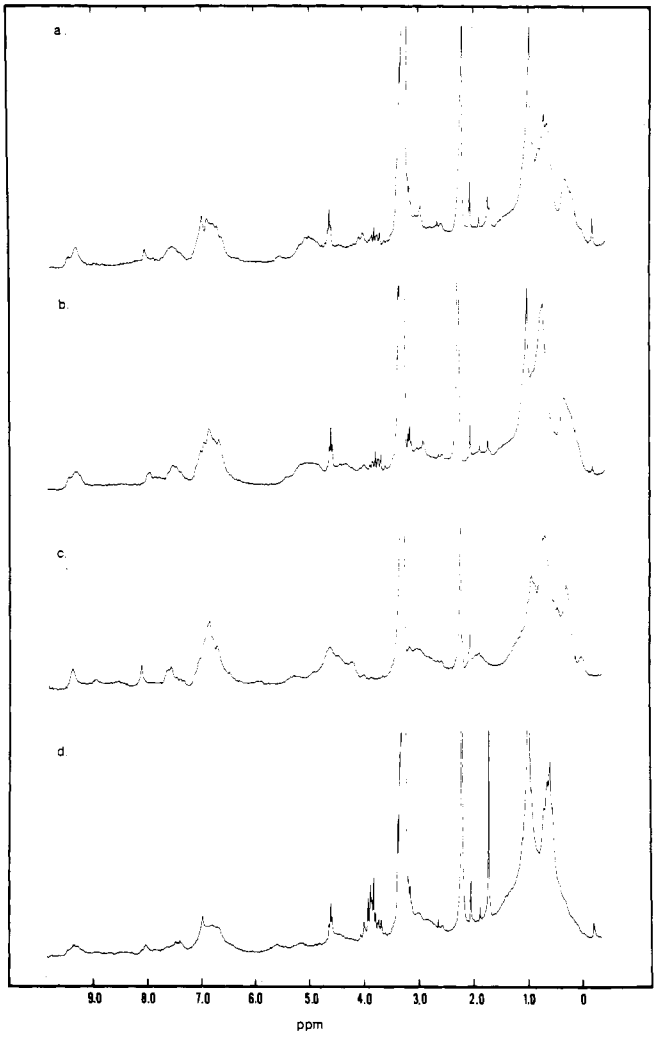

FIGURE 6: The ^{1}H nmr spectra of gramicidin isolated species in dioxane-d_8 (100 MHz). Species 1, 2, and 4 were prepared using the high yield protocols with the exchangeable hydrogens partially deuterated. Species 3 was obtained from crystals from CH_3OD. The following peaks are solvent contaminants: ethyl acetate (quartet near 4.0 ppm, singlet at 1.9 ppm, and triplet near 1.1 ppm); undeuterated dioxane at 3.6 ppm; and water at 2.4 ppm. (a) 1; (b) 2; (c) 3; (d) 4.

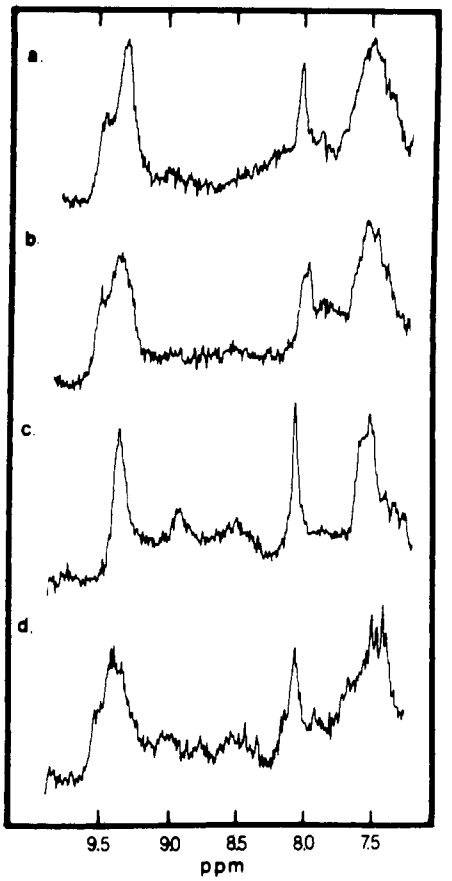

FIGURE 7: Expanded downfield region of ^{1}H nmr spectra of gramicidin isolated species in dioxane-d_8 (100 MHz). (a) 1; (b) 2; (c) 3; (d) 4.

and 4 are closely related and are qualitatively different from that of species 3. In the following discussion only dimer structures are considered for the isolated species. The data bearing on the molecular weight of the various species are discussed in the following paper (Veatch and Blout, 1974); the evidence supporting the dimer structure is strongest for species 3 and least strong for species 4.

Specific Models. Gramicidin has a strictly alternating LDLD sequence if glycine is considered to be an "honorary" D residue. The N-terminal is formylated, and the C-terminal is in an amide link with ethanolamine. Because of the limited amount of data available, only "regular structures," in which all LD dipeptides have the same conformation, will be considered here. The assumed regularity results in structures which are, in the broadest sense, helices.

Urry (1971) and Urry *et al.* (1971) have postulated a family of symmetrical π(LD) helical dimers as possible structures for the gramicidin transmembrane channel; Ramachandran and Chandrasekaran (1972) independently postulated a similar secondary structure, based primarily upon conformational energy considerations. In its hydrogen bonding the π(LD) helical monomer is essentially a rolled up parallel-β structure. All of the π(LD) helices, with about 4, 6, 8, or 10 residues per turn, were somewhat arbitrarily postulated to be left-handed, and the end-to-end ("head-to-head") dimer was postulated to involve the two N-formyl groups symmetrically in the antiparallel-β hydrogen bonding at the interface between the helices. For the present discussion we will consider both right- and left-handed π(LD) helices, as well as head-to-head, head-to-tail, and tail-to-tail aggregation.

A new family of double-helical dimers is postulated here to account for several properties of the isolated species seemingly not consistent with π(LD) helices. These include, in general, the very slow rates of aggregation and disaggregation in nonpolar solvents (see the following paper, Veatch and Blout, 1974) and, in particular for species 3, the 1680-cm^{-1} infrared amide I band which suggests largely antiparallel-β hydrogen bonding.

In these double-helical dimers the two chains are coiled about a common axis in a right- or left-handed helix. *A priori* there is no reason to favor one handedness over the other. All hydrogen bonds are intermolecular and are either parallel-β or entirely antiparallel-β. The antiparallel-β double helix is formally analogous to the DNA double helix with the two chains running in opposite directions and related by a twofold rotation axis perpendicular to the helix axis. Figure 8 is a schematic drawing of a left-handed antiparallel-β *double helix* with seven residues per turn (one chain is shown shaded, and the hydrogen bonds are denoted by dotted lines). All of the double-helical dimers have a cylindrical hole down the middle (>3 Å in diameter), as do the π(LD) helices with six or more residues per turn. The rise per turn of the double-helical dimers is about twice that of the π(LD) helical dimers of comparable diameter.

TABLE I: Differential Properties of Isolated Species.

Parameter	Species 1	Species 2	Species 3	Species 4
Nmr				
Formyl protons	Single peak	Doubled peak?	Single peak	Single peak?
Indole NH	*Identical* Major 9.4 ppm, minor 9.5 ppm		Major 9.4 ppm, minor 9.0 ppm	Major 9.4 ppm, minor 9.5 ppm
Methyl ring-current shift	*Identical* ~0.4 ppm		Major 0.4 ppm, minor 0.7 ppm	*No* resolved peak
Infrared				
Amide I	*Identical* Major 1633 cm^{-1}, shoulders 1650–1660 cm^{-1}		Major 1633 cm^{-1}, shoulder 1650 cm^{-1}, resolved 1680 cm^{-1}	Major 1633 cm^{-1}, shoulders 1650 cm^{-1}
CD				
Peptide 190–250 nm	*Identical* Negative 228 nm, negative 209 nm, positive 195 nm		Negative 228 nm, negative 220 nm, positive 194 nm	*Mirror image of 1 and 2* positive 228 nm, positive 209 nm, negative 195 nm
Aromatic 250–310 nm	Positive ^1L_a, positive ^1L_b		Positive ^1L_a, *No* ^1L_b	Positive ^1L_a, negative ^1L_b
Fluorescence[a]	*Identical*			
Polarization in ethyl acetate	0.019		0.025	0.019
Intensity change upon decay in ethanol	30%		0%	20%

[a] Data from following paper, Veatch and Blout, 1974.

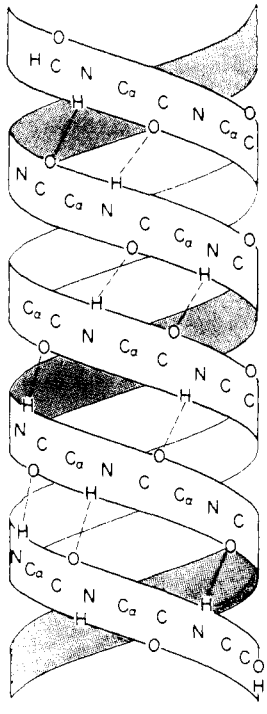

FIGURE 8: Schematic diagram of antiparallel-β double-helical gramicidin dimer with seven residues per turn and "even ends." The helix diagramed is left-handed; however, it is not now possible to specify the handedness of species 3. One chain is shaded and the other is not. The dotted lines denote hydrogen bonds. The formyl group of the unshaded chain is at the upper left, and the C-terminal hydroxyl of the unshaded chain is at the lower right. Bonds between atoms have been omitted because the geometry of the main-chain atoms is distorted for clarity.

The register of the two chains in the double-helical dimer is not uniquely determined by maximizing the number of hydrogen bonds. The two versions of the right-handed parallel-double-helical dimer with six residues per turn, differing in register, are shown in Figures 9a,b. The structure with "even ends" (a) has a strict twofold axis parallel to the helix axis relating the two monomers, while the alternate structure (b) lacks this strict symmetry axis.

Table II summarizes the parameters of the lower diameter parallel- and antiparallel-β double-helical dimers and compares them with those of the head-to-head π(LD) helical dimers, which are the same for either handedness. It should be noted that all of the structures in Table II have NH-C_αH dihedral angles near 180°. Also, all have ap-

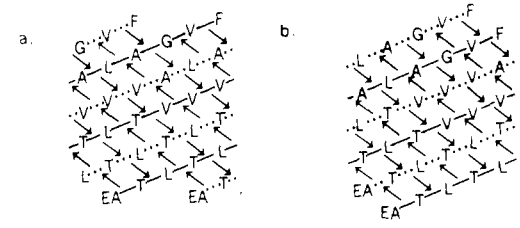

FIGURE 9: Schematic diagram of right-handed parallel-β double-helical gramicidin dimers with six residues per turn. These diagrams look at helices slit along the back and laid out flat. The letters denote the amino acid residues, and the two chains are shown as a solid line and dashed line, respectively. The arrows denote hydrogen bonds and go from CO to NH. In (a) the ends are "even," and the structure has a twofold rotation axis along the helix axis. In (b) the ends are not "even" and lack the formal twofold axis, but the total number of hydrogen bonds is the same as in (a).

TABLE II: Parameters of the Lower Diameter Parallel- and Antiparallel-β Double-Helical Dimers.

Parameter	Summary of Double-Helix Parameters[a]				Summary of Head-to-Head π(LD) Helical-Dimer Parameters		
	Parallel-β		Antiparallel-β				
Residues/turn	6	7	6	7	4.4[b]	6.3[b]	8.4[b]
Symmetry (orientation of rotation axis relative to helix axis)	C_2 or C_1 $\parallel$	C_1	C_2 $\perp$	C_2 $\perp$	C_2 $\perp$	C_2 $\perp$	C_2 $\perp$
Length, Å	32	25	32	28	37[b]	28[b]	21[b]
Rise/turn, Å	11	10	12	11	5.5	5.0	5.0
Inside diameter, Å	~3	~5	~3	~4	1.4[b]	4[b]	6[b]
Outside diameter, Å							
C-terminal	16–18	17–20	16	18–22			
N-terminal	10–13	14–16	Uniform	Uniform			
Hydrogen bonds (including hydroxyl)	30	28	30	28	Inter 4	6	8
					Intra 26	22	18
					Total 30	28	26

[a] Dimensions were measured from CPK models. [b] From Urry *et al.*, 1971.

proximately the same number of hydrogen bonds and would be stabilized by nonpolar solvents (see following paper, Veatch and Blout, 1974).

Conformational Conclusions

The conclusions are first stated here and are subsequently discussed in detail. The most probable regular structure for species 3 is one of the antiparallel-β double-helical dimers. This suggestion is supported by the infrared, formyl proton resonance, and X-ray diffraction data. It is doubtful whether any of the π(LD) dimers would have enough antiparallel-β hydrogen bonding to yield a resolved 1680 cm^{-1} band.

Species 1 and 4 could equally well be parallel double-helical dimers or π(LD) helical dimers. These suggestions are supported by the infrared and CD data. Species 4 would be composed of helices of opposite handedness to those of species 1. If species 2 is indeed asymmetric, as is suggested by the apparent doubling of the formyl proton resonance, then it must be an asymmetric parallel-β double-helical dimer or, less likely, a head-to-tail π(LD) helical dimer, closely related to species 1. The detailed interferences for species 1, 2, and 4 are discussed below separately from those for species 3.

Species 3. The amide I bands at 1633 and 1680 cm^{-1} observed for species 3 are within the range of values found for natural and synthetic L-polypeptides in the antiparallel-β pleated sheet conformation (Miyazawa and Blout, 1961), but the broad amide II band at 1545 cm^{-1} is outside the range (1520–1530 cm^{-1}). For the parallel-β pleated sheet of an L-polypeptide (β-keratin), no 1680-cm^{-1} amide I band is observed, and two amide II bands are observed (a strong band at 1530 cm^{-1} and a weak band at 1550 cm^{-1}) (Miyazawa and Blout, 1961) similar to the spectra of species 1, 2, and 4. To what extent will the positions and intensities of the bands change with deviations from the all-L pleated sheet geometry induced by the presence of an alternating LDLD sequence and the presence of a helix axis not along the chain direction? Miyazawa's (1960) perturbation treatment of the amide I and II frequencies, which successfully accounted for the bands of the α-helix, parallel, and antiparallel-β pleated sheet structures, can be extended to the π(LD) helices and double-helical dimers discussed earlier (Veatch, 1973). For the amide I transition frequencies, two sets are predicted—one identical with those of the pleated sheet conformation and the other dependent upon the details of the helix. It is possible to conclude that the amide I bands for species 3 imply largely antiparallel-β hydrogen bonding, such as the antiparallel-β double helix, while the amide I bands for species 1, 2, and 4 imply largely parallel-β hydrogen bonding, such as the parallel-β double helix or π(LD) helix. It seems unlikely that the single turn of antiparallel hydrogen bonding at the interface of the head-to-head or tail-to-tail π(LD) dimer would yield a resolved 1680-cm^{-1} amide I band.

The single formyl resonance observed in the ^1H nmr spectrum of species 3 is consistent with the presence of a twofold axis; all of the antiparallel-β double helices and head-to-head or tail-to-tail π(LD) helical dimers possess twofold axes.

Gramicdin crystallized from methanol has $P2_1$ symmetry (with two asymmetric units per unit cell, each containing a gramicidin dimer) with unit cell parameters a = 15 Å, b = 27 Å, c = 32 Å, and β = 92° (Veatch, 1973). If the dimer in the asymmetric unit is compact, its possible dimensions are 15 Å × 27 Å × 16 Å, 15 Å × 14 Å × 32 Å, or 8 Å × 27 Å × 32 Å. The first two possibilities are cylinders approximately 30 Å long. Cowan and Hodgkin (1953) noted evidence for helices in crystals of gramicidin B. There is little firm evidence to support the assumption that species 3 actually has a regular structure beyond the alternating LDLD sequence itself.

Because of the possibility of strong interactions among tryptophan transitions and those of the peptide backbone, the CD spectrum of species 3 cannot, at present, be used to specify a specific helix or handedness for this species.

Species 1, 2, and 4. In general, one would expect mirror image LD structures to have exactly mirror image CD spectra. The mirror image transformation not only interchanges handedness for helices, it also interchanges L and D for each residue. Thus, a right-handed α helix of an L-homopolypeptide would have a spectrum exactly mirroring that of a left-handed α helix of D-homopolypeptide but only an *approximate* mirror image of a left-handed α helix of an L-

homopolypeptide. It is reasonable to conclude that the observed approximate mirror image relationship of the CD spectra implies that the peptide backbone of species 1 and 2 is *approximately* the mirror image of the peptide backbone of species 4; for a regular structure these species would be helices of opposite handedness.

For gramicidin the D residues are not identical with the L residues. Energetically, changing the handedness of LDLD helix is equivalent to leaving the handedness unchanged and, instead, interchanging the L and D side chains. For both the π(LD) helices and the double helices, the C_α-C_β bond is approximately perpendicular to the helix axis for both L and D residues; therefore, interchanging the L and D residues might have only minor energetic consequences.

In principle, calculations of the CD spectra for each of the proposed structures could be used to determine the handedness and perhaps some details of the helix; however, in view of the high probability of large interaction among the transitions of the four tryptophan (whose conformations are unknown for the various models) and the peptide chromophores, such conclusions are not possible at present for any of the isolated species.

If a pure gramicidin dimer has nonequivalent chemical shifts for corresponding protons on the two constituent gramicidin monomers, then that dimer must be asymmetric. The observed broadening, or doubling, of the N-formyl proton resonance suggests that species 2 is asymmetric. However, it is also possible that species 2, as defined by the tlc preparation, might be a roughly equimolar mixture of two distinct conformational species with nearly equal tlc mobilities, both of which might be symmetric aggregates.

The infrared amide I and II bands of species 1, 2, and 4 are all very similar and are consistent with parallel-β double helices or π(LD) helical dimers (see discussion under species 3).

One of the difficulties with the postulation of a head-to-tail aggregation of π(LD) helices is the tendency of such systems to aggregate indefinitely. It is hard to imagine a mechanism whereby dimers would be formed and in which trimers, tetramers, etc., with similar kinetic stability would not be present at a somewhat higher concentration of total gramicidin. In particular, each one of the isolated species can be dried into a solid and redissolved without yielding additional species as assayed by tlc. Only a symmetric dimer, such as the head-to-head π(LD) dimer, or a "closed" dimer, such as the double helices, would not aggregate further.

The substitution of a methyl group for the gramicidin formyl proton in *N*-acetyldesformylgramicidin does not appreciably destabilize any of the isolated species in ethanol solution (see following paper, Veatch and Blout, 1974); following the reasoning of Urry (1971), this result would be consistent with none of the isolated species being head-to-head π(LD) dimers.

Since species 1 and 2 have such similar secondary structure but are separated on tlc, it is necessary to postulate that they differ in some detail, such as the register of the double helical dimers or the head-to-head *vs.* tail-to-tail of the π(LD) dimers. In the following paper (Veatch and Blout, 1974) evidence is presented which suggests that species 1 and 2 are *not* a dimer-tetramer or monomer-dimer pair; however, direct molecular weight data for these two species would be needed to prove this conclusively. The possibility that the structures of species 1 and 2 could differ in the number of residues per turn and still yield the observed nearly identical CD spectra cannot be excluded.

Summary

Four conformational species of gramicidin were physically isolated from a single organic solvent system. Two classes of conformations were found and correlated with regular dimer models. (1) Species 1, 2, and 4 are concluded to be helical structures with predominantly parallel-β hydrogen bonding. Species 1 and 2 are very similar, while species 4 has an opposite helical sense from species 1 and 2. Species 1, 2, and 4 may be either parallel-β double-helical dimers or π(LD) helical dimers. (2) Species 3 has predominantly antiparallel hydrogen bonding and may have a symmetric structure. Of the regular structures considered, species 3 is most likely an antiparallel-β double helix.

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