Conformation of the Gramicidin A Channel in Phospholipid Vesicles: A Fluorine-19 Nuclear Magnetic Resonance Study†

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Received January 3, 1985

ABSTRACT: The membrane conformation of the peptide ionophore gramicidin A is shown by ^{19}F NMR to be described by the N-terminal to N-terminal β_{LD} helical dimer model proposed by Urry [Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676]. Fully active analogues of gramicidin with ^{19}F labels at both the N- and C-termini are prepared synthetically. Labeled peptides are incorporated into small unilamellar vesicles of dimyristoylphosphatidylcholine. Measurements of the accessibility of the labels to either aqueous or lipophilic paramagnetic probes show that the N-terminus of gramicidin is located in the membrane interior and the C-terminus is at the membrane surface. Of the specific models proposed for the structure of gramicidin, these data are consistent only with that of Urry. The C-terminal ^{19}F NMR peak in vesicles actually consists of three overlapping peaks. Experiments with the aqueous shift reagent Tm^{3+} show that C-terminal ^{19}F nuclei in the inner and in the outer leaflets of vesicles resonate at different frequencies. The outer leaflet peak in turn consists of two overlapping peaks, possibly due to a local rearrangement of the C-terminal label.

Gramicidin A is a linear pentadecapeptide that forms transmembrane channels specific for monovalent cations. [For a recent review, see Andersen (1984)]. The simplicity of its structure makes it an experimentally approachable system for understanding the molecular basis for ion transport across biological membranes. The first requirement for such studies is determination of the conformation of the gramicidin channel.

Gramicidin is composed of alternating D- and L-amino acids, and its N- and C-termini are blocked by a formyl and ethanolamine group, respectively. The amino acid sequence of the major form, [Val]gramicidin A, is HCO-L-Val_1-Gly_2-L-Ala_3-D-Leu_4-L-Ala_5-D-Val_6-L-Val_7-D-Val_8-L-Trp_9-D-Leu_{10}-L-Trp_{11}-D-Leu_{12}-L-Trp_{13}-D-Leu_{14}-L-Trp_{15}-$NHCH_2CH_2OH$ (Sarges & Witkop, 1965a).[1] The conducting gramicidin channel is a dimer (Tosteson et al., 1968; Goodall, 1970; Urry et al., 1971; Kolb et al., 1975; Veatch et al., 1975; Bamberg & Janko, 1977; Veatch & Stryer, 1977). Two classes of models have been proposed for the conformation of gramicidin in solution and in the membrane, on the basis of conductance studies of chemically modified gramicidins and on the basis of spectroscopic data. These are shown schematically in Figure 1. Models A and B are single-helical channels dimerized end to end in the membrane interior. Models C and D are double helices in which both peptide chains span the membrane. Model A is the N-terminal to N-terminal β_{LD} helical dimer first proposed by Urry (1971; Urry et al., 1971; then called the π^6(L,D) helix). Model B is an analogous C-terminal to C-terminal dimer suggested by Bradley et al. (1978). Model C represents the antiparallel β double helix shown by Veatch et al. (1974) to exist in gramicidin solutions in nonpolar organic solvents, such as dioxane. Model D is the corresponding parallel β double helix.

The four models differ in the location of their N- and C-termini in bilayer systems. Only dimer A has its N-termini buried in the membrane interior. Only dimer B has its C-termini buried in the membrane interior. Only dimers C and D have both their N- and C-termini exposed at the membrane surface. Thus, we can choose between these models if we can locate both the N- and C-termini to either the membrane surface or the membrane interior.

In this paper we determine which of these four models represents the conformation of the gramicidin channel in lipid bilayers by using NMR spectroscopy[2] to locate the N- and C-termini. In outline, we first identified the N- and C-terminal resonances in the NMR spectrum of gramicidin incorporated into phosphatidylcholine vesicles.[3] We then perturbed the

† This work was supported, in part, by U.S. Public Health Service Grants AI-15311, AM-07300, and AM-10794, as well as grants from the Pharmaceutical Manufacturers Association, the Milton Fund, and the Medical Foundation. J.T.D. was supported by a U.S. Public Health Service training grant (T32-GM-07598). W.R.V. was the recipient of a U.S. Public Health Service career development award (AI-00320). NMR experiments were performed at the NMR Facility for Biomolecular Research at the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139, which is supported by Grant RR-00995 from the Division of Research Resources of the National Institutes of Health.

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[1] In the naturally occurring variants gramicidin B and gramicidin C, the L-Trp residue in position 11 is replaced by L-Phe and L-Tyr, respectively. Additionally, 5–20% of all naturally occurring gramicidins have an L-Ile residue in place of L-Val in position 1 (Sarges & Witkop, 1965b). Throughout this paper, we will refer to the naturally occurring mixture of these six components simply as "gramicidin".

[2] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; HPLC, high-performance liquid chromatography; UV, ultraviolet; gramicidin, the naturally occurring mixture of [Val]- and [Ile]-gramicidins A, B, and C; phosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoylphosphatidylcholine; N-^{19}F-gramicidin, [4-fluoro-L-Phe^1]gramicidin; N,C-^{19}F-gramicidin, *O*-(*p*-fluorobenzoyl)[4-fluoro-L-Phe^1]gramicidin; C-^{19}F-gramicidin, *O*-(*p*-fluorobenzoyl)gramicidin; Me_2SO-d_6, perdeuterated dimethyl sulfoxide; TFA, trifluoroacetic acid.

[3] From simultaneous conductance and fluorescence measurements of a fluorescent gramicidin derivative in planar bilayers, Veatch et al. (1975) showed that all gramicidin dimers are conducting channels. Veatch & Stryer (1977) used fluorescence energy transfer to show that gramicidin dimerizes in phosphatidylcholine vesicles. Hence, the incorporation of gramicidin into phosphatidylcholine vesicles offers a convenient system for the study of the gramicidin channel conformation in a membrane environment.

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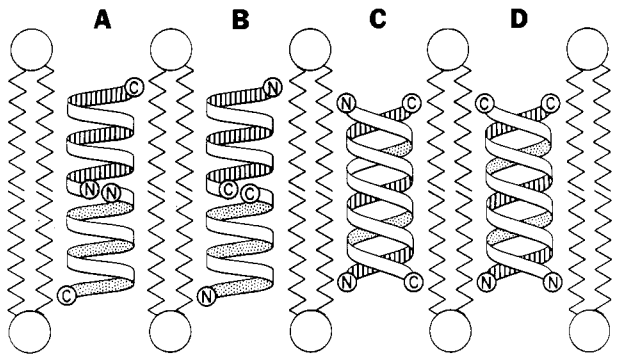

FIGURE 1: Models proposed for the conformation of the gramicidin transmembrane channel. This diagram depicts a lipid bilayer membrane containing four different gramicidin dimer channel conformations. The gramicidin monomer polypeptide chain is represented as a ribbon with the N- and C-termini labeled. Note that one ribbon in each dimer has vertical stripes. All models are helical with roughly the same size hole down the middle through which ions and water may pass. Model A is the N-terminal to N-terminal β_{LD} model of Urry (1971), model B is a C-terminal to C-terminal dimer of the same β_{LD} helix considered by Bradley et al. (1978), models C and D are the antiparallel β and parallel β double helices of Veatch et al. (1974), respectively.

spectrum with paramagnetic probes whose location was known (Bystrov et al., 1971). These probes act by dipolar interactions and so have a greater effect on nuclei closer to them (Bergelson, 1978; Weinstein et al., 1980). Thus, we determined whether given nuclei were closer to the membrane interior or to the surface. As noted, this determination for both the N- and C-termini suffices to determine the channel conformation of gramicidin.

The use of ^{1}H and ^{13}C NMR to study membrane proteins is limited by several factors: viz., (a) the concentration of protein in the membrane is low, (b) wide resonance peaks result from the immobility of the proteins, and (c) the many resonances from lipid nuclei cover the NMR spectra of complex systems. Fluorine-19 NMR offers several advantages to offset these limitations. The sensitivity of the ^{19}F nucleus is only slightly lower than that of ^{1}H. There is no interference from lipid resonances because fluorine is not a constituent of biological membranes. Chemical shifts and spin–lattice relaxation times are potentially more sensitive to the chemical environment for ^{19}F than for either ^{1}H or ^{13}C because of the chemical shift anisotropy relaxation mechanism. Finally, a covalently bound fluorine atom is nearly isosteric with a hydrogen atom (Goldman, 1969). Therefore, in our studies we used a gramicidin derivative labeled with fluorine atoms at both the N- and C-termini (Figure 2).

We perturbed the spectra with three paramagnetic probes: the ion Tm^{3+}, an upfield shift reagent; the ion Mn^{2+}, a relaxation reagent; and a nitroxide spin-label, which acted in these experiments as a relaxation reagent. The first two were located in the aqueous phase. In particular, since gramicidin transports only monovalent cations, neither of these had access to the membrane interior. The spin-label was attached near the methyl end of a fatty acid chain, and so buried in the membrane interior. A preliminary account of this work has appeared (Weinstein et al., 1979).

Experimental Procedures

Materials. Gramicidin was obtained from ICN and was purified by crystallization from 95% ethanol. Acetic anhydride, aniline, dimethylformamide, and methanol were all distilled before use. Pyridine and triethylamine were distilled over calcium hydride. The following chemicals were used as purchased: formic acid (95–97%) and *p*-fluorobenzoyl chloride

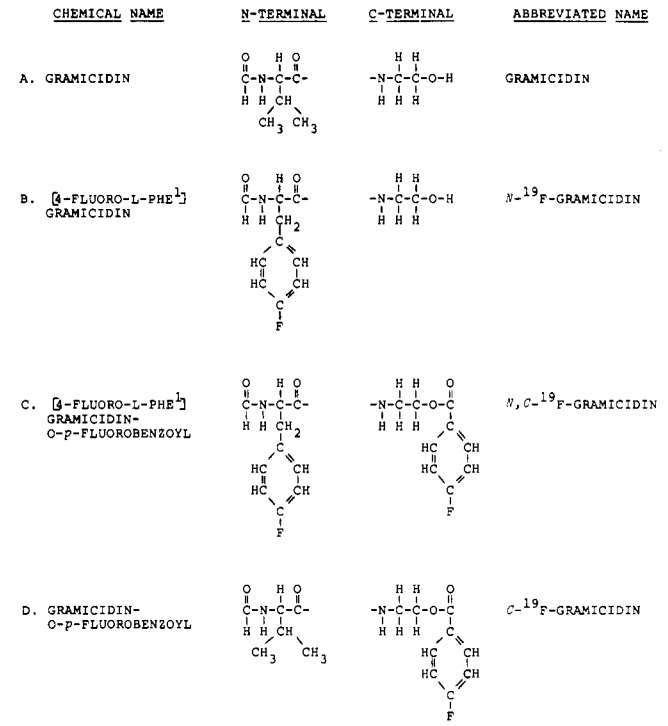

FIGURE 2: Chemical structure and nomenclature of ^{19}F-labeled gramicidin derivatives. Only the first amino acid residue is indicated at the N-terminus and only the ethanolamide and its esters at the C-terminus. The intervening 14 amino acids are not shown.

(98%), Aldrich; DMPC (synthetic, A grade), Calbiochem; dicyclohexylcarbodiimide, Eastman; phenyl isothiocyanate (sequenal grade), Pierce; 4-fluoro-DL-phenylalanine and 1-hydroxybenzotriazole, Sigma; 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy, Syva. Cation-exchange resin (AG-MP-50, 100–200 mesh) was from Bio-Rad Laboratories.

Analytical Methods. UV spectra were measured in methanol on a Cary 15 spectrophotometer. For gramicidin and derivatives, concentrations were measured by UV absorbance at 290 nm (ϵ = 22 400 cm^{-1} M^{-1}). The absorbance at 320 nm relative to that at 290 nm (A_{320}/A_{290}) was taken as a criterion of purity (theoretical value zero).

For amino acid analysis, 0.1 μmol of gramicidin or its derivatives in methanol solution was evaporated to dryness in ampules. It was then dissolved in 40 μL of acetic acid and 0.2 mL of 6 N HCl containing 2% phenol. The ampules were degassed, sealed, and heated to 116 °C for 72 h. The hydrolysis solution was evaporated to dryness and submitted for amino acid analysis.

Synthesis of Formyl-4-fluoro-L-phenylalanine. 4-Fluoro-DL-phenylalanine was enzymatically resolved and the L enantiomer purified according to Tong et al. (1971). The optical purity was determined by a gas chromatographic method, as described by Beitler & Feibush (1976). The preparation contained less than 0.5% D enantiomer. The resolution factor [(retention time of D isomer)/(retention time of L isomer)] of a DL mixture was found to be 1.22 with complete base-line separation.

4-Fluoro-L-phenylalanine (2 mmol) was dissolved in 5 mL of formic acid and cooled in an ice bath. Acetic anhydride (1.75 mL) was added. After 30 min in the ice bath, the mixture was kept at room temperature for 60 min. Water was added (3.0 mL), and the solution was evaporated to dryness. The product was purified on a cation-exchange column (H^+ form, 50 × 1.1 cm) eluted with methanol. Thin-layer chromatography on silica gel plates in acetic acid–chloroform 2:1

Table I: Amino Acid Analysis of Gramicidin Analogues (Expected over Result)[a]

amino acid	crystallized gramidicin mix	des(formylvalyl)-gramicidin	semisynthetic [Val^1]gramicidin	N-^{19}F-gramicidin	N,C-^{19}F-gramicidin
Val	3.86 2.36	3 2.88	4 3.87	3 2.95	3 2.47
Ile	0.14 0.12	0 ND[b]	0 ND[b]	0 ND[b]	0 ND[b]
Gly	1 1.06	1 1.10	1 1.01	1 1.04	1 1.02
Ala	2 2.08	2 1.91	2 2.00	2 1.96	2 2.01
Leu	4 3.60	4 3.76	4 3.96	4 3.92	4 3.90
Trp[d]	3.83 2.73	3.83 2.82	3.83 3.13	3.83 2.73	3.83 2.80
Phe	0.03 trace[c]	0.03 ND[b]	0.03 trace[c]	0.03 trace[c]	0.03 0.04
Tyr	0.14 0.12	0.14 0.11	0.14 0.08	0.14 0.11	0.14 0.15
4-fluoro-Phe	0 ND[b]	0 ND[b]	0 ND[b]	1 0.95	1 0.64

[a] Expressed as moles of amino acid per mole of peptide. Expected value printed directly above each experimental value. Moles of peptide calculated as the average of moles of Gly, (moles of Ala)/2, and (moles of Leu)/4. [b] ND, not detectable (less than about 0.01 mol/mol of peptide). [c] Trace, a just detectable but unquantifiable peak. [d] Values for Trp uncorrected for losses during hydrolysis.

(v/v) visualized by Cl_2–tolidine gave a single spot of R_f 0.8. The absence of free amino acid was confirmed by dansylation (Weiner et al., 1972). ^{19}F and ^{1}H NMR spectra were taken on a Brüker HX 270 spectrometer and were in accord with the desired structure.

Synthesis of Des(formylvalyl)gramicidin. Two grams of gramicidin (1.1 mmol) was desformylated according to Sarges & Witkop (1965a), using HCl gas dried by passage through concentrated sulfuric acid. The product was applied in methanol to a cation-exchange column (H^+ form, 50 × 2.5 cm) and eluted at 4 °C with concentrated ammonium hydroxide in methanol (1:4 v/v). The product was then chromatographed in methanol on a Sephadex LH-20 column (25–100-μm particle size, 150 × 4 cm). Column chromatography was monitored by UV absorbance at 290 nm. Pooling fractions from the center of the elution profile yielded 1.6 g of desformylgramicidin (80% yield).

This product was dissolved in 90 mL of pyridine and 5 mL of triethylamine. The flask was wrapped in aluminum foil, and the solution was washed with argon dried by a concentrated sulfuric acid trap. Phenyl isothiocyanate (5 mL) was added. The flask was closed tightly and held at 42 °C for 4.5 h. Aniline was then added (3.5 mL), and after 10 min the solution was evaporated to near dryness. The vacuum was released by N_2. The residue was dissolved in 200 mL of 1.5 N HCl in methanol, left at room temperature for 1 h, evaporated to dryness, and washed with absolute ethanol. The residue, a viscous syrup, was mixed with methanol to give a heavy white crystalline precipitate; this was filtered and triturated with methanol. The solution was applied to a Sephadex LH-20 column (150 × 4 cm) and eluted with methanol. The peak fractions were pooled and applied to a cation-exchange column (H^+ form, 50 × 2.5 cm), washed with methanol, and eluted with 2 N ammonia in methanol. The yield was 1.06 g (70%).

The amino acid analysis of des(formylvalyl)gramicidin is given in Table I. The ratio $A(320)/A(290)$ was less than 0.001. The product was also characterized by coupling formyl-L-valine according to Weinstein et al. (1980) to form semisynthetic [Val^1]gramicidin. The latter product was characterized by amino acid analysis (Table I), UV spectrophotometry, and ^{13}C NMR. All were in accord with the proposed structure.

Semisynthetic [Val^1]gramicidin was further characterized by HPLC. The chromatogram of natural gramicidin is shown in Figure 3A. The six peaks were assigned to the six components according to Axelsen & Vogelsang (1977). This assignment fit well the known percentage composition of the six components as well as their relative hydrophobicities. The chromatogram of semisynthetic [Val^1]gramicidin is shown in Figure 3B. Since the N-terminal isoleucine or valine residues of the several isomers were removed by the Edman degradation and all replaced by valine, only three components are expected in this chromatogram. However, other products (indicated by arrows) appeared in the chromatograms of all gramicidin derivatives synthesized from des(formylvalyl)gramicidin. Their position in the chromatogram depended on their N-terminal amino acid, but did not change upon acylation of the C-terminal ethanolamide hydroxyl (compare, e.g., chromatograms C and D of Figure 3). This demonstrates that the side products were generated by blocking of the C-terminal hydroxyl groups during Edman degradation. In each case, they constitute less than 15% of the product.

Synthesis of [4-Fluoro-L-Phe^1]gramicidin (N-^{19}F-gramicidin, Figure 2B). Des(formylvalyl)gramicidin and 4-fluoro-L-phenylalanine (50 μmol each in 0.5 mL of dimethylformamide) were coupled by a 20% excess of dicyclohexylcarbodiimide in the presence of 1 equiv of 1-hydroxybenzotriazole. The reaction mixture was held at 4 °C for 48 h. It was chromatographed on a cation-exchange column (H^+ form, 20 × 1.1 cm) eluted with methanol, followed by a Sephadex LH-20 column (120 × 1.1 cm) eluted with methanol. The yield was 80%. The product was characterized by amino acid analysis (Table I) and HPLC (Figure 3C). The ratio $A(320)/A(290)$ was less than 0.001. Elemental analysis for fluorine found 0.96% (expected 0.97%).

Synthesis of O-(p-Fluorobenzoyl)[4-fluoro-L-Phe^1]-gramicidin (N,C-^{19}F-gramicidin, Figure 2C). N-^{19}F-gramicidin (20 μmol) was dissolved in 0.5 mL of pyridine, and 10 μL of *p*-fluorobenzoyl chloride was added, giving a 4:1 molar ratio acyl chloride:peptide. The reaction mixture was kept at room temperature for 2 h and then evaporated to dryness under

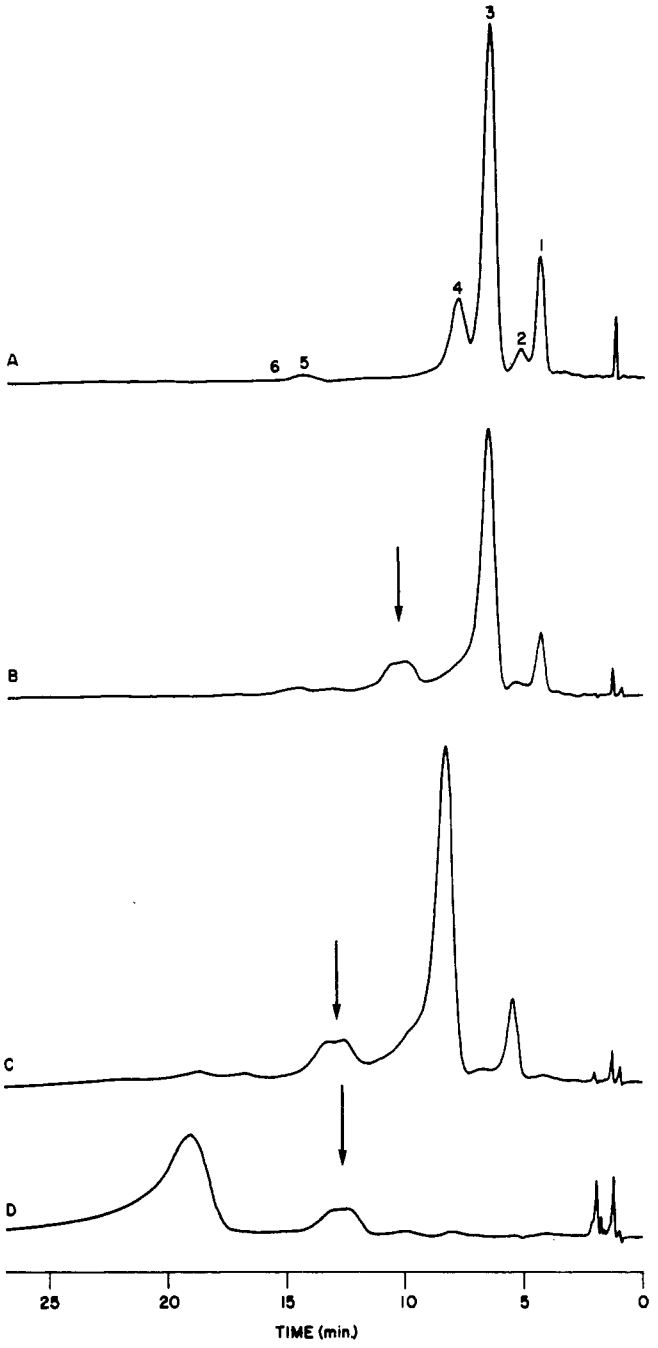

FIGURE 3: HPLC chromatograms of (A) gramicidin. The naturally occurring variants are identified by the numerals 1–6: (1) [Val]-gramicidin C; (2) [Ile]gramicidin C; (3) [Val]gramicidin A; (4) [Ile]gramicidin A; (5) [Val]gramicidin B; (6) [Ile]gramicidin B. (B) Semisynthetic [Val^1]gramicidin, (C) N-^{19}F-gramicidin, and (D) N,C-^{19}F-gramicidin. The arrows in (B–D) denote the side product discussed under Experimental Procedures. HPLC was performed on a Zorbax-C8 column (Du Pont Instruments) attached to a Waters Associates high-pressure liquid chromatograph. All chromatograms were run isocratically in 80% methanol/20% water (v/v) at a flow rate of 3 mL/min (3000 psi). Detection was by UV absorbance at 280 nm.

vacuum without heating. Methanol was added and the solution reevaporated; this was repeated 4 times. The product was chromatographed on a Sephadex LH-20 column (120 × 1.1 cm) eluted with methanol. The purified product was characterized by amino acid analysis (Table I) and by HPLC (Figure 3D). The ratio $A(320)/A(290)$ was 0.03. Elemental analysis for fluorine found 1.86% (expected 1.83%).

Synthesis of O-(p-Fluorobenzoyl)gramicidin (C-^{19}F-gramicidin, Figure 2D). This compound was synthesized exactly as N,C-^{19}F-gramicidin, except that the starting material was gramicidin. Elemental analysis for fluorine found 0.78%, four-fifths the expected value of 0.96%. High-pressure liquid chromatograms (not shown) were also consistent with a mixture of 80% C-^{19}F-gramicidin and 20% unreacted gramicidin. The ester linkage between gramicidin and the *p*-fluorobenzoyl moiety is expected to be somewhat unstable in polar solvents. Since unmodified gramicidin is silent in a ^{19}F NMR experiment, this preparation was used without further purification.

Preparation of Vesicles. Small unilamellar vesicles of DMPC and gramicidin (or gramicidin derivative) were prepared by sonication as described previously (Weinstein et al., 1980). The molar ratio peptide:lipid was 1:30, and the concentration of peptide was 5 mM. Sonication was usually performed under N_2. However, when this precaution was omitted, no change was noted in the resultant spectra.

For experiments involving salts present only outside the vesicles, concentrated salt solutions were added to the desired final concentration. For experiments involving salts both inside and outside the vesicles, the sample was resonicated for 5 min. For experiments in which Tm^{3+} was present only inside the vesicles, the external Tm^{3+} was removed after sonication by four rounds of dialysis of at least 4 h each. In the first round, the sample was dialyzed against 1000 volumes of 40 mM EDTA to ensure removal of Tm^{3+} bound to the lipid head group. In the second and third rounds, the sample was dialyzed against 1000 volumes of 40 mM $MgCl_2$ and, in the final round, against 20 volumes of 40 mM $MgCl_2$ in D_2O. Since Tm^{3+} is trivalent and gramicidin transports only monovalent cations, the only route by which it can escape from the vesicles is leakage through the bilayer. This is so slow as to be undetectable in our experiments.

Spin-labeled vesicles were made as above with the addition of 2 mol % spin-labeled lipid. Spin-labeled lipid was prepared and purified as in Weinstein et al. (1980). Briefly, a nitroxide spin-label was chemically bound to carbon number 16 of stearic acid to form 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy (Syva spin-label 616). The spin-labeled fatty acid was then introduced into position 1 of phosphatidylcholine molecules with myristic acid in position 2. Control vesicles in the spin-label experiments contained 2 mol % 1-stearoyl-2-myristoylphosphatidylcholine.

Extraction of Gramicidin. Gramicidin was extracted from DMPC vesicles into Me_2SO-d_6, a solvent in which phosphatidylcholines are insoluble. Vesicles with gramicidin were made as described above. Water was then removed by lyophilization. The residue was dissolved in 1 mL of absolute ethanol and allowed to stand for 15 min to disperse the lipid and the peptide. One milliliter of Me_2SO-d_6 was added and mixed. The ethanol was removed by evaporation with a stream of N_2, precipitating the DMPC. The mixture was then filtered with suction through Whatman No. 1 filter paper, and the filtrate was washed with a small amount of Me_2SO-d_6. Concentration of gramicidin in the Me_2SO phase was measured by absorbance at 290 nm; percent recovery could then be calculated.

Nuclear Magnetic Resonance Measurements. NMR experiments were conducted on a Brüker HX270 spectrometer operating at 254.135 MHz for ^{19}F and utilized pulsed Fourier-transform methodology with quadrature detection. Sample volumes were generally 0.4 mL, contained in a spinning 5 mm o.d. Wilmad NMR tube with a Teflon plug for vortex suppression. The experiments that examined the multiple C-terminal peaks used sample volumes of 1.5 mL contained in a spinning 10 mm o.d. Wilmad NMR tube. Temperature was

controlled to ±1 °C by the Brüker variable-temperature controller. All spectra were recorded with broad-band proton decoupling and with internal D_2O or Me_2SO-d_6 lock.

Chemical shifts are expressed relative to external 10% trifluoroacetic acid in D_2O.[4] Chemical shifts in Me_2SO-d_6 are uncorrected for the change in lock frequency. In the Tm^{3+} experiments of Weinstein et al. (1980), it was observed that the Tm^{3+} caused an upfield shift in the D_2O lock frequency relative to the terminal methyl groups of the DMPC fatty acid chains. This shift in lock frequency was 16, 24, and 37 Hz for Tm^{3+} to gramicidin molar ratio of 1:1, 2:1, and 4:1, respectively. All these shifts were added to the corresponding ^{19}F chemical shifts measured relative to the deuterium lock. T_1 measurements were made by the inversion–recovery method as described in detail in Weinstein et al. (1980).

Results

The doubly labeled derivative N,C-^{19}F-gramicidin retained 15% of the gross conductance activity of gramicidin in planar bilayers (Weinstein et al., 1979). The single-channel characteristics of the singly labeled derivative N-^{19}F-gramicidin have recently been measured by Koeppe et al. (1984). The single-channel conductance at 25–50 mV in 1.0 M NaCl is 5.8 pS, compared with 12.4 pS for [Val]gramicidin A. The mean channel lifetime is 100–200 ms, compared with 500 ms for [Val]gramicidin A. These two factors alone quantitatively account for the decrease in gross conductance of N,C-^{19}F-gramicidin, consistent with the observation that esterifications to the C-terminal ethanolamine do not drastically affect the activity of gramicidin (Apell et al., 1977; Weinstein et al., 1980; Whaley et al., 1985). Therefore, N,C-^{19}F-gramicidin forms active channels, and study of its membrane conformation is relevant to the question of the conformation of native gramicidin.

The ^{19}F NMR spectrum of N,C-^{19}F-gramicidin in Me_2SO-d_6 had, as expected, two peaks (Weinstein et al., 1979). The C-terminal peak was at −28.00 ppm and was 16 ± 2 Hz wide. The N-terminal peak was at −38.82 ppm and was 11 ± 2 Hz wide.[4] Peak assignments were made by comparisons with the spectrum of the singly labeled compound, N-^{19}F-gramicidin.

The spectrum of N,C-^{19}F-gramicidin in DMPC vesicles (Weinstein et al., 1979) had a broad N-terminal peak, about 220 Hz wide, at −40.32 ppm. The C-terminal peak appeared as two overlapping peaks, at −29.46 and −29.99 ppm, of composite width 270 Hz. These line widths were comparable to the 254-MHz ^{19}F line widths measured by Dettman et al. (1984) for 3-fluorophenylalanine and 3-fluorotyrosine incorporated into a membrane-bound protein.

The multiple C-terminal peaks were not due to loss of chemical integrity. C-^{19}F-gramicidin, incorporated into DMPC vesicles by sonication, was extracted into Me_2SO-d_6; 55% of the peptide was recovered in the Me_2SO phase. The NMR spectrum of this solution had a single peak, of the same chemical shift and width as C-^{19}F-gramicidin dissolved in Me_2SO-d_6. Recoveries of 90% could be achieved by repeated extractions, but at the cost of 5-fold dilution. In this case the NMR spectrum had a single peak, of the same width but 0.1 ppm upfield from that of C-^{19}F-gramicidin (not shown). We believe this shift was a concentration effect; the important point is that only a single peak was still seen. Together these results argue that (i) there was only a single chemically distinct C-terminal fluorine after sonication and (ii) it was the same fluorine as before sonication. It will be shown below that the structure of the C-terminal peak was due to incorporation of the labeled peptide into small unilamellar vesicles.

[4] In the preliminary paper of this work (Weinstein et al., 1979), fluorine chemical shifts relative to TFA were systematically reported 39.26 ppm downfield of their true values. That error is corrected in this paper. The conclusions in the previous paper depend only on *changes* in chemical shift, and so are unaffected by this error.

Table II: Tm^{3+}-Induced Changes in Chemical Shift ($\Delta\delta$, ppm)[a]

	1:1[b]	2:1[b]	4:1[b]
N-terminal peak	0.11	0.19	0.28
C-terminal peak[c]	0.49	0.75	1.01

[a] N,C-^{19}F-gramicidin in DMPC vesicles. Changes in chemical shift are relative to zero Tm^{3+} and are corrected for change in lock frequency as described under Experimental Procedures. All changes are upfield. Shift reagent is external to vesicles. Spectra acquired at 50 °C with 4096 data points per spectrum, 5000-Hz spectral width, 8000–10 000 acquisitions, and 10-Hz exponential line broadening. [b] Molar ratio, shift reagent:peptide. [c] After addition of $TmCl_3$, C-terminal peak structure was not resolved.

Table III: T_1 Values and Rate Enhancement by Paramagnetic Probes[a]

	T_1 control (s)	T_1 paramagnetic (s)	rate enhancement (s^{-1})
	Mn^{2+}[b]		
N-^{19}F-gramicidin	0.59[c]	0.50[c]	0.29[c]
N,C-^{19}F-gramicidin			
N-terminal	0.74	0.49	0.69
C-terminal	0.44	0.053	17
	Spin-Labeled Lipid[d]		
N-^{19}F-gramicidin	0.64	0.070	13
N,C-^{19}F-gramicidin			
N-terminal	0.62	0.13	6.0
C-terminal	0.47	0.19	3.1

[a] Gramicidin analogues in DMPC vesicles. T_1 was measured by inversion–recovery. Rate enhancement was calculated as $(T_1 \text{ paramagnetic})^{-1} - (T_1 \text{ control})^{-1}$. Spectra were acquired at 50 °C with 4096 data points per spectrum, ±1000 to ±2500 Hz spectral width, 1.0–1.5-s recycle time, 3000–4500 acquisitions, and 5–20-Hz exponential line broadening. [b] Paramagnetic condition: 10 mM $MnCl_2$. Control: 10 mM $MgCl_2$. [c] This experiment at 40 °C. [d] Paramagnetic condition: vesicles of DMPC + 2% spin-labeled lipid. Control: vesicles of DMPC + 2% 1-stearoyl-2-myristoylphosphatidylcholine.

Thulium Ion Induced Chemical Shifts. The upfield shift reagent Tm^{3+} was added to vesicles containing N,C-^{19}F-gramicidin, and the changes in chemical shift of the N- and C-terminal resonances were measured (Table II). The N-terminal signal hardly moved, while the C-terminal signal moved upfield appreciably. (At this resolution, splitting in the C-terminal peak could not be resolved after addition of Tm^{3+}.) No change in chemical shift was observed upon addition of Tm^{3+} to vesicles containing N-^{19}F-gramicidin. Therefore, the C-terminal fluorine was accessible at the membrane surface, but the N-terminal fluorine was not.

Relaxation Enhancement by $MnCl_2$. The spin–lattice relaxation times, T_1, were measured for gramicidin derivatives in vesicles in the presence of Mg^{2+} and in the presence of the paramagnetic ion Mn^{2+}, and the rate enhancement was calculated (Table III). Only the C-terminal fluorine was greatly influenced by the paramagnetic NMR probe. The N-terminal fluorine was not significantly influenced in either the singly labeled or the doubly labeled derivatives. This confirmed that the C-terminal fluorine was accessible at the membrane surface, but the N-terminal fluorine was not.

Relaxation Enhancement by Nitroxide Spin-Label. Spin–lattice relaxation times were measured for gramicidin derivatives in spin-labeled vesicles and in control vesicles. The influence of the spin-label was greatest at the N-terminal fluorine (Table III).

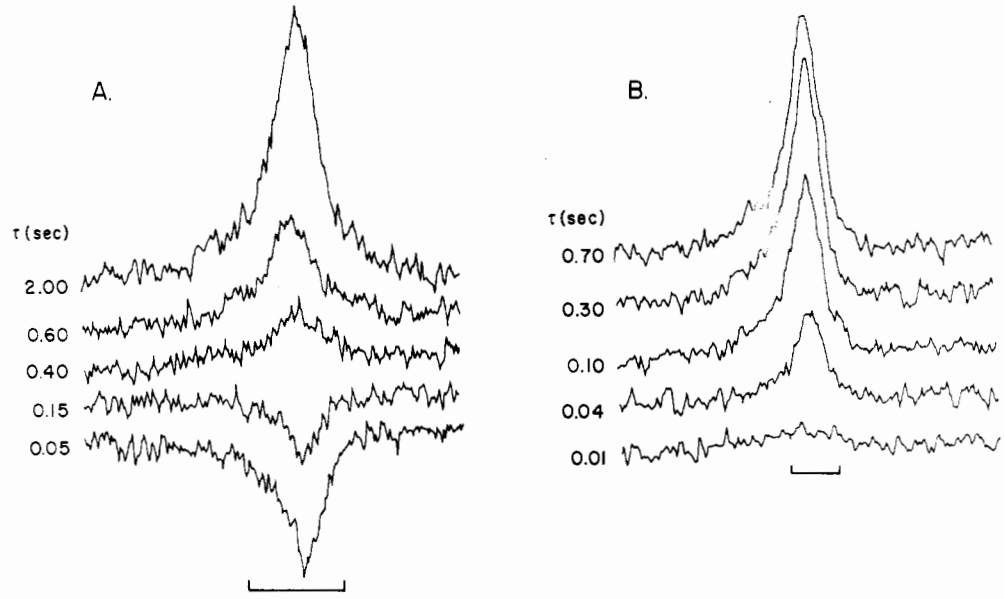

FIGURE 4: Inversion–recovery experiments for N-^{19}F-gramicidin in DMPC vesicles in the presence of 1 equiv (5 mM) of $MgCl_2$. The variable delay (s) between the 180° (inversion) pulse and the 90° (acquisition) pulse is indicated for each spectrum. Scale bars equal 1.0 ppm. (A) Control vesicles (2% 1-stearoyl-2-myristoylphosphatidylcholine) at 40 °C: 204 data points, ±1000-Hz spectral width, 1.5-s recycle time, 3400 acquisitions, and 5-Hz exponential line broadening. (B) Spin-labeled vesicles (2% spin-labeled lipid) at 50 °C: 4096 data points, ±2000-Hz spectral width, 1.0-s recycle time, 4000 acquisitions, and 20-Hz exponential line broadening.

Figure 4 depicts a relaxation time experiment by the inversion–recovery method (180°–τ–90°–acquisition time–pulse delay) for N-^{19}F-gramicidin in DMPC vesicles containing 2 mol % control lipid (Figure 4A) or 2 mol % spin-labeled lipid (Figure 4B). Fluorine in the spin-labeled vesicles relaxed much faster than in vesicles without a paramagnetic probe: the magnetization was already positive at τ = 0.01 s in the presence of the paramagnetic group, whereas it was positive only after τ = 0.15 s in the control experiment. These data show that the N-terminal fluorine was accessible in the membrane interior, but the C-terminal fluorine was not.

C-Terminal Peak Structure. An expanded NMR spectrum resolved the C-terminal peak into three overlapping peaks: a closely spaced pair at −29.37 and −29.55 ppm and a much broader peak at −29.99 ppm (Figure 5B). The relative areas of the downfield pair to the broad upfield peak were about 60:40. This is the surface area ratio of the outer to the inner leaflet of small unilamellar vesicles determined by a variety of techniques (Johnson et al., 1975; Hutton et al., 1977). Identical nuclei can have different chemical shifts in the inner and outer leaflets of small unilamellar vesicles (see Discussion). We therefore tested whether this accounted for the C-terminal peak structure by preparing vesicles with the upfield shift reagent Tm^{3+} sealed inside.

If the dialysis to remove external Tm^{3+} was performed at 23 °C, the two downfield peaks were not appreciably shifted, but the broad upfield peak was shifted further upfield by 0.1 ppm (Figure 5A). (In this experiment, chemical shifts were not corrected for the Tm^{3+}-induced change in lock frequency. Since Tm^{3+} was sealed inside the vesicles and the inner volume of these small unilamellar vesicles was only about 5% of the total, this correction would have been negligible.) If the dialysis was performed at 30 or 37 °C, spectra were consistent with a larger shift in the upfield peak position, 0.3 ppm or more (not shown; the signal-to-noise ratio was considerably worse for these spectra). These data showed that the broad upfield peak was accessible to internal Tm^{3+}. The data in Table II showed that the two downfield peaks were accessible to external Tm^{3+}. Therefore, the downfield pair and the upfield peak represented nuclei in the outer and inner leaflets, respectively, of the vesicle bilayer.

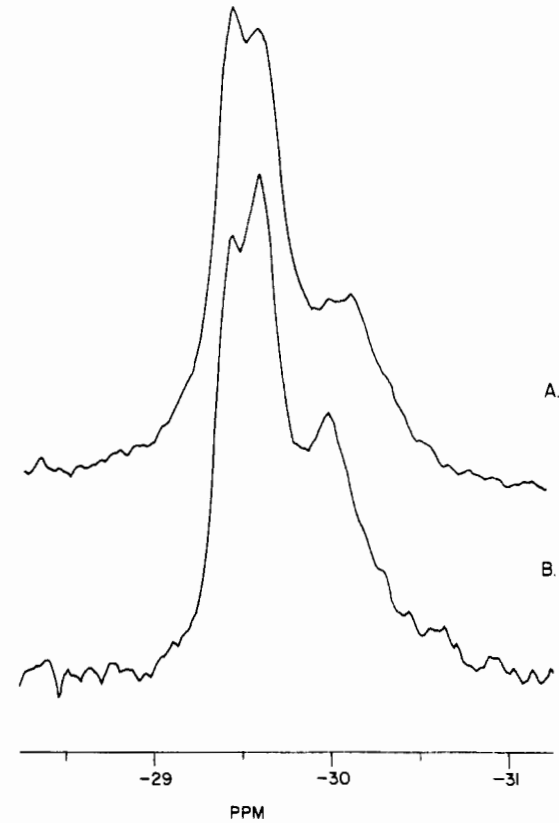

FIGURE 5: Expansion of 254-MHz ^{19}F NMR spectra of C-^{19}F-gramicidin in DMPC vesicles. Spectra acquired at 52 °C with 4096 data points, 10000-Hz spectral width, 1.0-s recycle time, and 10-Hz exponential line broadening. Chemical shift in ppm relative to 10% trifluoroacetic acid in D_2O (external). (A) $TmCl_3$ (40 mM) sealed inside for a molar ratio shift reagent:peptide of 8:1, 6144 acquisitions. (B) No additions, 4096 acquisitions.

Peak structure was also examined by external titration of vesicles with small amounts of Tm^{3+} (Figure 6). Since the broad upfield peak was not accessible externally, in this experiment it was considered a chemical shift standard. The

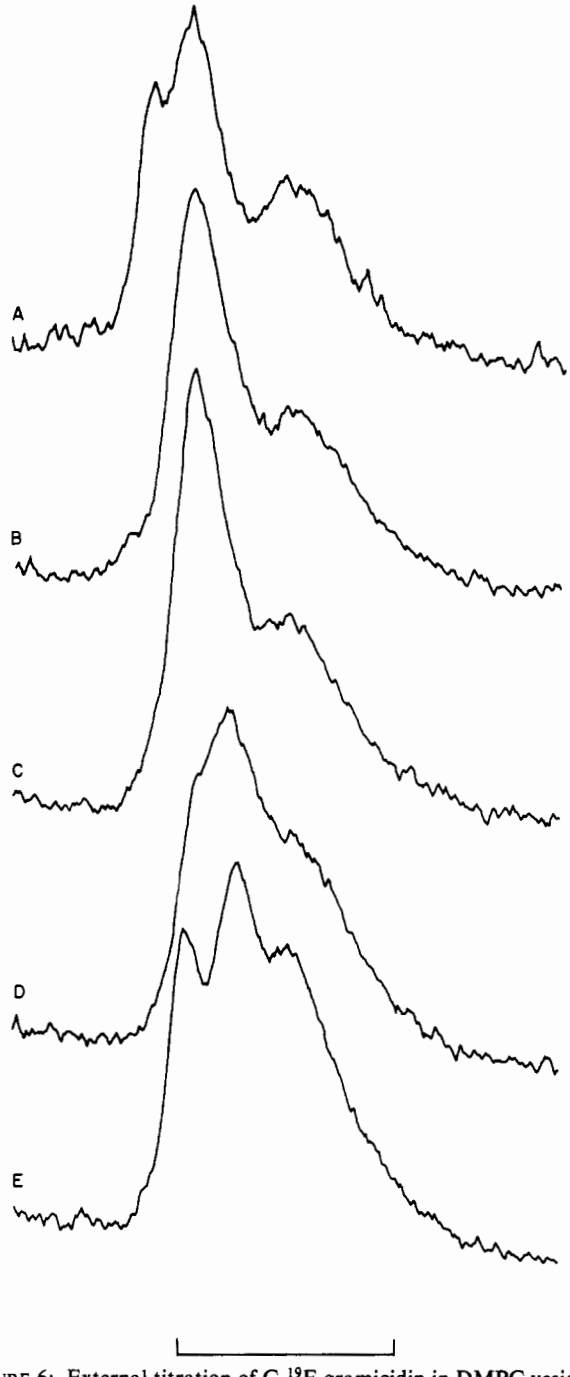

FIGURE 6: External titration of C-^{19}F-gramicidin in DMPC vesicles with $TmCl_3$. Spectra were aligned visually by the inner leaflet peak at −29.99 ppm, which is inaccessible to the shift reagent. The 254-MHz ^{19}F spectra were acquired at 52 °C, with 4096 data points, 2000-Hz spectral width, 2.0-s recycle time, and 4-Hz exponential line broadening. Scale bar equals 1.0 ppm. Molar ratios of Tm^{3+}:peptide were (A) 0.0:1 (2048 acquisitions), (B) 0.22:1 (4096 acquisitions), (C) 0.44:1 (4096 acquisitions), (D) 0.66:1 (4096 acquisitions), and (E) 0.88:1 (5120 acquisitions).

downfield peak shifted through the peak that had been in the middle before addition of shift reagent. Throughout the titration, the chemical shift difference between this latter peak and the broad upfield peak remained unchanged. Therefore, of the two peaks arising from the outer leaflet, the downfield peak was more accessible to the aqueous shift reagent Tm^{3+}.

Discussion

The results with Tm^{3+} and Mn^{2+} demonstrate that the C-terminus is accessible at the surface of the membrane but the N-terminus is not. The lipid spin-label results demonstrate that the N-terminus is accessible in the membrane interior but the C-terminus is not. These results exclude the C-terminal to C-terminal helical dimer and the antiparallel and parallel β double helices and strongly favor the N-terminal to N-terminal helical dimer (Figure 1A) as the major conformation of the gramicidin channel in phosphatidylcholine vesicles.

Feigenson et al. (1977) reported 80-MHz ^{1}H NMR spectra of gramicidin incorporated into perdeuterated DMPC vesicles. These workers detected the aromatic proton resonances and concluded from experiments with aqueous shift reagents that these protons were accessible to solvent. These spectra did not, however, resolve any N-terminal resonances. Because we have determined the location of both termini for the same molecules in a single experiment, we can draw a strong conclusion about channel conformation.

The spectra obtained with Tm^{3+} sealed inside vesicles demonstrate that the C-terminal fluorines have different chemical shifts in the inner and outer leaflets of small unilamellar vesicles. Such a frequency shift is observed in high-field ^{1}H NMR spectra of small unilamellar phosphatidylcholine vesicles for the choline methyl resonance (Sheetz & Chan, 1972; Kostelnik & Castellano, 1973) and, at higher fields yet (400–500 MHz), for the acyl chain resonances (Brouillette et al., 1982; Schuh et al., 1982). The frequency of the phosphate resonance from phosphatidylcholine vesicles is also shifted between the inner and outer leaflets in ^{31}P NMR spectra at about 40 MHz (Berden et al., 1974, 1975; Uhing, 1975), although at higher fields (129 MHz) chemical shift anisotropy broadens the resonance sufficiently to obscure the shift (Berden et al., 1974). Schmidt et al. (1977) observe a similar shift in the 25-MHz ^{13}C spectrum of vesicles of 1,2-dipalmitoylphosphatidylcholine specifically ^{13}C enriched in the carbonyl carbon. In all these cases the inner leaflet peak is upfield of the outer. This is in the same direction as the ^{19}F shift observed in the present work. The shift has most reasonably been attributed to greater electronic shielding at the inner leaflet as a consequence of the higher density of phospholipid head groups (Berden et al., 1975; Hutton et al., 1977; Brouillette et al., 1982).

[Longmuir & Dahlquist (1976) reported a similar shift in the 94-MHz ^{19}F spectrum of vesicles made from specific gem-difluoro derivatives of 1,2-distearoylphosphatidylcholine. In this case, the inner leaflet peak was upfield. The shift was attributed here to rapid trans–gauche isomerization, with different average conformations in the two leaflets.]

In the experiments with Tm^{3+} sealed inside vesicles, the inner leaflet peak shifts less than expected for the amount of Tm^{3+} added. We considered at first the possibility that much of the Tm^{3+} leaked out of the vesicle during the prolonged dialysis. However, Lichtenberg et al. (1979) also reported that shift reagents have less effect at the inner leaflet of small unilamellar vesicles than at the outer. They suggested that this is also due to the higher density of head groups on the inner leaflet, which blocks the shift reagent.

We suggest that the two outer membrane peaks at −29.37 and −29.55 ppm are due to a slow equilibrium between two conformations of the label, one more accessible to the aqueous phase than the other (Figure 7). The extra peak is not due to another peptide conformation with the C-terminus buried in the membrane, since none of the C-terminal fluorine is left behind in titrations with higher concentrations of Tm^{3+}. Furthermore, only a single C-terminal peak was observed in the ^{13}C version of this experiment (Weinstein et al., 1980). The titration with small concentrations of Tm^{3+} (Figure 6)

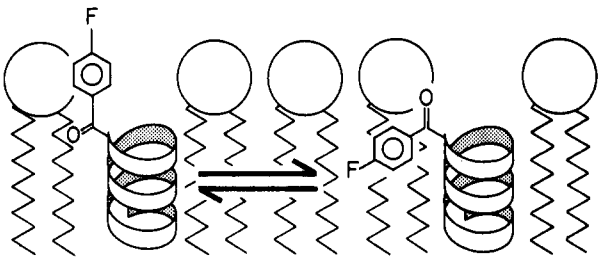

FIGURE 7: Rearrangement of C-terminal fluorine label proposed to account for the spectral shift in the outer leaflet NMR peak. This diagram depicts half a lipid bilayer containing gramicidin monomers, depicted as ribbons as in Figure 1, with two possible conformations of the *O*-(*p*-fluorobenzoyl) moiety.

shows that the downfield peak is more accessible to the external aqueous phase. Evans (1960) showed that ^{19}F chemical shifts lie at lower fields in solvents of higher polarizability. Therefore, the frequencies of these two peaks are in the right direction to be a solvent shift.

The inner leaflet resonance was a single peak. This may be because label in the inner leaflet cannot rearrange as proposed in Figure 7, due to the higher density of phospholipid head groups. Alternatively, it may simply be because the inner leaflet peak is so broad that the frequency shift is not resolved. We cannot distinguish between these possibilities.

With the work presented here and in previous publications (Weinstein et al., 1979, 1980), there are now three independent lines of evidence for the membrane conformation of the gramicidin channel. All three point toward the N-terminal to N-terminal helical dimer proposed by Urry (1971).

The second line of evidence consists of the variety of data on channel-forming activity of gramicidin derivatives in planar bilayers. Minor modifications of the N-terminus, such as elimination of the formyl moiety (Morrow et al., 1979) or its replacement with an acetyl moiety (Szabo & Urry, 1979), have drastic effects on channel formation. Conversely, extensive modifications of the C-terminus (Apell et al., 1977) do not. These results are explicable in terms of the N-terminal to N-terminal helical dimer, but not in terms of either of the double helices, in which both termini are located at the channel mouth.

The third line of evidence is the ^{13}C NMR data of Urry et al. (1983). They synthesized a series of gramicidins specifically ^{13}C enriched in a single carbonyl, incorporated them into 2-lysophosphatidylcholine suspensions, and measured the change in chemical shift induced by binding of Tl^+. The results are explicable in terms of the N-terminal to N-terminal helical dimer but are not sensible in terms of either the parallel or the antiparallel double helices.

(A conflicting line of evidence is the infrared spectra of gramicidin in 1,2-dipalmitoylphosphatidylcholine vesicles (Ovchinnikov & Ivanov, 1983). These authors claim that the channel conformation is the antiparallel β double helix. While we would be delighted if this were the case, as this structure was first proposed in our laboratory (Veatch et al., 1974), we have some reservations about their conclusion. Their infrared measurements are technically demanding: they obtained peptide spectra at a peptide:lipid molar ratio of 1:300, while other workers [e.g., Cortijo et al. (1982)] have obtained reliable peptide spectra only with great difficulty at a molar ratio of 1:30. Their conclusions about the solution conformations of gramicidin appear to be inconsistent with two-dimensional NMR data of Arseniev et al. (1984). Furthermore, from independent infrared measurements and normal mode calculations, Naik & Krimm (1984) recently have concluded that the channel conformation is the N-terminal to N-terminal helical dimer).

We need no longer be content to study only the effect of a polypeptide on the NMR spectrum of the lipid. It is now possible to study membrane-bound peptides directly with NMR techniques (Feigenson et al., 1977; Wallace & Blout, 1979; Sugihara et al., 1982; Braun et al., 1983; Urry et al., 1983; Deber & Behnam, 1984; Dettman et al., 1984; Keniry et al., 1984). Greater sensitivity can be obtained by using more sophisticated NMR instrumentation and techniques, such as magic-angle sample spinning (Harbison et al., 1984), and by isotopic enrichment of the peptide or depletion of the lipid. The results presented here suggest that NMR techniques will be particularly useful for determining which regions of the polypeptide chain of a membrane protein lie in the membrane interior and which are outside the membrane.

ACKNOWLEDGMENTS

We thank Paul Gallop and Peter Hauschka for the amino acid analyses, Olaf S. Andersen, David E. Golan, and Barbara J. Jackson for critical comments on the manuscript, and Eliezar Dawidowicz, Laura Green, Shripad Kelkar, and Bonnie Wallace for helpful discussions. We also thank David Ruben and Ron Haberkorn of the National Magnet Laboratory for their heroic efforts with a recalcitrant fluorine probe.

Registry No. DMPC, 13699-48-4; gramicidin A, 11029-61-1; formyl-4-fluoro-L-phenylalanine, 39064-03-4; 4-fluoro-L-phenylalanine, 1132-68-9; desformylgramicidin A, 63808-16-2; des[formyl(Val^1)]-gramicidin A, 71388-81-3; [Val^1]gramicidin A, 4419-81-2; [4-fluoro-L-Phe^1]gramicidin A, 71967-40-3; *O*-(*p*-fluorobenzoyl)[4-fluoro-L-Phe^1]gramicidin, 72032-60-1; *p*-fluorobenzoyl chloride, 403-43-0; *O*-(*p*-fluorobenzoyl)gramicidin A, 96482-80-3.

REFERENCES

Andersen, O. S. (1984) *Annu. Rev. Physiol. 46*, 531–548.
Apell, H.-J., Bamberg, E., Alpes, H., & Läuger, P. (1977) *J. Membr. Biol. 31*, 171–188.
Arseniev, A. S., Bystrov, V. F., Ivanov, V. T., & Ovchinnikov, Y. A. (1984) *FEBS Lett. 165*, 51–56.
Axelsen, K. S., & Vogelsang, S. H. (1977) *J. Chromatogr. 140*, 174–178.
Bamberg, E., & Janko, K. (1977) *Biochim. Biophys. Acta 465*, 486–499.
Beitler, U., & Feibush, B. (1976) *J. Chromatogr. 123*, 149–166.
Berden, J. A., Cullis, P. R., Hoult, D. I., McLaughlin, A. C., Radda, G. K., & Richards, R. E. (1974) *FEBS Lett. 46*, 55–58.
Berden, J. A., Barker, R. W., & Radda, G. K. (1975) *Biochim. Biophys. Acta 375*, 186–208.
Bergelson, L. D. (1978) *Methods Membr. Biol. 9*, 275–335.
Bradley, R. J., Urry, D. W., Okamoto, K., & Rapaka, R. (1978) *Science (Washington, D.C.) 200*, 435–437.
Braun, W., Wider, G., Lee, K. H., & Wuthrich, K. (1983) *J. Mol. Biol. 169*, 921–948.
Brouillette, C. G., Segrest, J. P., Ng, T. C., & Jones, J. L. (1982) *Biochemistry 21*, 4569–4575.
Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I., & Bergelson, L. D. (1971) *Chem. Phys. Lipids 6*, 343–350.
Cortijo, M., Alonso, A., Gomez-Fernandez, J. C., & Chapman, D. (1982) *J. Mol. Biol. 157*, 597–618.
Deber, C. M., & Behnam, B. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 61–65.
Dettman, H. D., Weiner, J. H., & Sykes, B. D. (1984) *Biochemistry 23*, 705–712.

Evans, D. F. (1960) *J. Chem. Soc. 1960*, 877–880.
Feigenson, G. W., Meers, P. R., & Kingsley, P. B. (1977) *Biochim. Biophys. Acta 471*, 487–491.
Goldman, P. (1969) *Science (Washington, D.C.) 164*, 1123–1130.
Goodall, M. C. (1970) *Biochim. Biophys. Acta 219*, 471–478.
Harbison, G. S., Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Lugtenburg, J., Herzfeld, J., Mathies, R., & Griffin, R. G. (1984) *Biochemistry 23*, 2662–2667.
Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19*, 255–265.
Johnson, L. W., Hughes, M. E., & Zilversmit, D. B. (1975) *Biochim. Biophys. Acta 375*, 176–185.
Keniry, M. A., Kintanar, A., Smith, R. L., Gutowsky, H. S., & Oldfield, E. (1984) *Biochemistry 23*, 288–298.
Koeppe, R. E., II, Andersen, O. S., & Mazet, J.-L. (1984) *International Biophysics Congress Abstracts, 8th*, p 273 (Abstr.), Heisn Conference Services, Petersfield, Great Britain.
Kolb, H.-A., Läuger, P., & Bamberg, E. (1975) *J. Membr. Biol. 20*, 133–154.
Kostelnik, R. J., & Castellano, S. M. (1973) *J. Magn. Reson. 9*, 291–295.
Lichtenberg, D., Amselem, S., & Tamir, I. (1979) *Biochemistry 18*, 4169–4172.
Longmuir, K. J., & Dahlquist, F. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2716–2719.
Morrow, J. S., Veatch, W. R., & Stryer, L. (1979) *J. Mol. Biol. 132*, 733–738.
Naik, V. M., & Krimm, S. (1984) *Biochem. Biophys. Res. Commun. 125*, 919–925.
Ovchinnikov, Y. A., & Ivanov, V. T. (1983) in *Conformation in Biology* (Srinivasan, R., & Sarma, R. H., Eds.) pp 155–174, Adenine Press, Guilderland, NY.
Sarges, R., & Witkop, B. (1965a) *J. Am. Chem. Soc. 87*, 2011–2020.
Sarges, R., & Witkop, B. (1965b) *Biochemistry 4*, 2491–2494.
Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1977) *Biochemistry 16*, 3948–3954.
Schuh, J. R., Banerjee, U., Muller, L., & Chan, S. I. (1982) *Biochim. Biophys. Acta 687*, 219–225.
Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.
Sugihara, T., Blout, E. R., & Wallace, B. A. (1982) *Biochemistry 21*, 3444–3452.
Szabo, G., & Urry, D. W. (1979) *Science (Washington, D.C.) 203*, 55–57.
Tong, J. H., Petitclerc, C., D'Iorio, A., & Benoiton, N. L. (1971) *Can. J. Biochem. 49*, 877–881.
Tosteson, D. C., Andreoli, T. E., Tieffenberg, M., & Cook, P. (1968) *J. Gen. Physiol. 51*, 373S–384S.
Uhing, M. C. (1975) *Chem. Phys. Lipids 14*, 303–308.
Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676.
Urry, D. W., Goodall, M. C., Glickson, J. D., & Mayers, D. F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1907–1911.
Urry, D. W., Trapane, T. L., & Prasad, K. U. (1983) *Science (Washington, D.C.) 221*, 1064–1067.
Veatch, W., & Stryer, L. (1977) *J. Mol. Biol. 113*, 89–102.
Veatch, W. R., Fossel, E. T., & Blout, E. R. (1974) *Biochemistry 13*, 5249–5256.
Veatch, W. R., Mathies, R., Eisenberg, M., & Stryer, L. (1975) *J. Mol. Biol. 99*, 75–92.
Wallace, B. A., & Blout, E. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1775–1779.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247*, 3242–3251.
Weinstein, S., Wallace, B. A., Blout, E. R., Morrow, J. S., & Veatch, W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4230–4234.
Weinstein, S., Wallace, B. A., Morrow, J. S., & Veatch, W. R. (1980) *J. Mol. Biol. 143*, 1–19.
Whaley, W. L., Paczkowski, J. A., Koeppe, R. E., II, Weiss, J., & Andersen, O. S. (1985) *Biophys. J. 47*, 431a (Abstr.).