Gramicidin-Induced Hexagonal H_{II} Phase Formation in Erythrocyte Membranes

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ABSTRACT: Using ^{31}P nuclear magnetic resonance (NMR), small-angle X-ray scattering (SAXS), and freeze–fracture electron microscopic (FFEM) techniques, it is shown that gramicidin induces a hexagonal H_{II} phase not only in liposomes prepared from total lipids extracted from human erythrocytes but also in isolated human erythrocyte membranes (white ghosts). A 37 °C, H_{II} phase formation is detected at a gramicidin to phospholipid molar ratio exceeding 1:80. At a molar ratio of 1:5, about 30% of the phospholipid is organized in the H_{II} phase. The gramicidin-induced H_{II} phase exhibits a very small ^{31}P chemical shift anisotropy [(CSA) $\sim 10 \pm 1$ ppm], indicating decreased head-group order, and it displays a temperature-dependent increase in tube diameter from 60.2 Å at 4 °C to 64.2 Å at 37 °C in ghosts and from 62.8 to 69.4 Å at 37 °C in total lipid extracts, both in the presence of 1 mol of gramicidin/10 mol of phospholipid. This anomalous temperature-dependent behavior is probably due to the presence of cholesterol. ^{31}P NMR data indicate that the H_{II} phase formation by gramicidin is temperature dependent and show the gradual disappearance of the H_{II} phase at low temperatures (<20 °C), resulting in a bilayer type of ^{31}P NMR line shape at 4 °C, whereas SAXS and FFEM data suggest equal amounts of H_{II} phases at all temperatures. This apparent discrepancy is probably the result of a decrease in the rate of lateral diffusion of the membrane phospholipids which leads to incomplete averaging of the ^{31}P CSA in the H_{II} phase. The induction of the H_{II} phase by gramicidin is specific in that N-formylation of the four tryptophan residues of gramicidin completely blocks the hexagonal (H_{II}) phase inducing ability of the peptide. Furthermore, the striking parallel between hexagonal H_{II} phase induction and the enhancement of lysophosphatidylcholine and palmitoylcarnitine transbilayer reorientation by gramicidin as well as the lack of effect of the formylated gramicidin as described in the preceding paper [Classen, J., Haest, C. W. M., Tournois, H., & Deuticke, B. (1987) *Biochemistry* (preceding paper in this issue)] strongly suggests the H_{II} phase formation and flip enhancement are mechanistically related phenomena. It is suggested that the formation of gramicidin aggregates of specific structure which are intermediates in H_{II} phase formation [Killian, J. A., & De Kruijff, B. (1985) *Biochemistry 24*, 7881–7890] leads to enhancement of transbilayer reorientation of phospholipids.

It is generally accepted that the majority of the lipids in biomembranes are organized in an extended bilayer (Singer & Nicolson, 1972). Although such an organization clearly explains the main membrane function, namely, acting as a selectively permeable barrier, there are several vital membrane processes such as membrane fusion, transbilayer movements of lipids, and protein insertion and translocation which require local and transient departures from the lipid bilayer structure and therefore are difficult to reconcile with a continuous bilayer organization. In this respect, considerable attention is given recently to the ability of lipids to adopt nonbilayer organizations. It should be noticed that a number of major membrane lipids adopt the nonlamellar H_{II}[1] phase when isolated and hydrated under physiological conditions (Cullis & De Kruijff, 1978; De Kruijff et al., 1980; Cullis et al., 1980). Since related stable nonlamellar structures have been observed in biological membranes (Coreless & Costello 1981; Larsson et al., 1985; Kachar & Reese, 1982) and processes such as those mentioned above are in agreement with the occurrence of short-living nonlamellar structures, inverted micellar or tubular lipid organizations of the type found in lipidic particles (Verkleij et al., 1979) and the H_{II} phase are most likely involved in membrane organization and function [for recent general overviews on lipid polymorphism in relation to membrane function, see De Kruijff et al. (1985) and Cullis et al. (1986)]. In view of the dynamic membrane processes, it can be expected that mechanisms exist which regulate lipid structure and the local occurrence of nonbilayer lipid organizations in the membrane. The most obvious candidates to function as modulators of lipid structure are membrane proteins and peptides. Gramicidin, a hydrophobic linear pentadecapeptide which has been widely used as a model for the membrane-spanning segment of integral membrane proteins (Rice & Oldfield, 1979; Chapman et al., 1979), turned out to be an attractive model to study peptide-induced modulation of lipid structure in model membrane systems [see Killian and De Kruijff (1986) for a recent review]. In lysophosphatidylcholines, it triggers a micellar to lamellar transition (Killian et al., 1983); in phosphatidylethanolamines, it lowers the bilayer to hexagonal H_{II} phase transition temper-

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[1] Abbreviations: CSA, chemical shift anisotropy; DMSO, dimethyl sulfoxide; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; FFEM, freeze–fracture electron microscopy; GR, gramicidin; H_{II}, hexagonal phase of type II; HPLC, high-performance liquid chromatography; NFG, tryptophan-N-formylated gramicidin; NMR, nuclear magnetic resonance; PL, phospholipid; SAXS, small-angle X-ray scattering; Tris, tris(hydroxymethyl)aminomethane; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole.

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ature (Van Echteld et al., 1981); and in phosphatidylcholines (Van Echteld et al., 1982) as well as in negatively charged phospholipids (Killian et al., 1986), it is able to induce the formation of an H_{II} organization.

The question we will address in this paper is whether gramicidin is able to modulate lipid structure in complex lipid systems as found in biomembranes. For this, we selected the erythrocyte membrane because of its relatively simple and well-characterized membrane organization.

Using ^{31}P nuclear magnetic resonance (NMR), small-angle X-ray scattering (SAXS), and freeze–fracture electron microscopy (FFEM), we demonstrate a concentration-dependent induction of a hexagonal H_{II} phase by gramicidin in dispersions of total lipid of erythrocytes and in erythrocyte ghosts. The effect is specific for gramicidin in that formylation of the four tryptophans of the molecule gives a total loss of the H_{II} phase induction. These observations will be discussed in light of the ability of the peptide to (specifically) increase the rate of transbilayer movement of certain lipids in the erythrocyte membrane as described in the preceding paper (Classen et al., 1987).

Materials and Methods

Gramicidin, natural mixture from *Bacillus brevis*, was purchased from Sigma Chemical Co. (St. Louis, MO) and was used without further purification. *N,N'*-Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBT), and *N*-methylmorpholine were obtained from Aldrich (Beerse, Belgium). Sodium [^{14}C]formate (specific activity 2.11 GBq/mmol) was obtained from Amersham (Buckinghamshire, U.K.). All solvents were of analytical grade. Methanol, dichloromethane, and *N,N*-dimethylformamide were freshly distilled before use.

Desformylgramicidin was prepared and purified as described by Killian et al. (1986). [^{14}C]Gramicidin (specific activity 2.11 GBq/mmol) was synthesized by reformylation of desformylgramicidin with sodium [^{14}C]formate. Ten milligrams of dry desformylgramicidin was dissolved in 300 μL of dichloromethane/dimethylformamide, 2:1 (v/v), containing 1-hydroxybenzotriazole, *N,N'*-dicyclohexylcarbodiimide, and *N*-methylmorpholine, each in a 3-fold molar excess with respect to desformylgramicidin. This mixture was added to 0.3 mg of sodium [^{14}C]formate, and the reaction was allowed to proceed for 18 h at 4 °C. Following acidification by addition of an equal volume of ice-cold 5 M HCl in methanol, the mixture was applied to a Dowex 50 W-X2 resin, 200–400 mesh (Merck, Darmstadt, FRG), column (60 × 7 mm) in the H^+ form equilibrated with methanol. The reformylated desformylgramicidin was eluted with 15 mL of methanol. After being dried, the product was dissolved in methanol and applied to an Si 100 Polyol RP 18 (5-μm particles; Serva Heidelberg, FRG) HPLC column (250 × 22 mm) and eluted with methanol/water, 83:17 (v/v), using a linear flow rate of 5 mL/min. All gramicidin-containing fractions (detection at 280 nm) were pooled and dried. To remove traces of impurities originating from the HPLC column, the product was finally applied onto a Sephadex LH-20 (Pharmacia, Sweden) column (500 × 20 mm) and eluted with methanol at a linear flow rate of 5.0 mL/min. Under these conditions, the yield of the highly pure [as judged from thin-layer chromatography and HPLC according to Killian et al. (1986)] gramicidin (specific activity 2.11 GBq/mmol) was approximately 30%.

Trp-N-formylated gramicidin was obtained from gramicidin according to the method described by Killian et al. (1985). [^{14}C]Trp-N-formylated gramicidin (specific activity 1.01 MBq/mmol) was obtained from [^{14}C]gramicidin with a recovery of approximately 80%, by the same method.

Preparation of Erythrocyte Ghost Membranes. Human erythrocytes were isolated from outdated blood by centrifugation (10 min, 3000*g*), and the buffy coat was removed. Cells were washed 3 times in 150 mM NaCl, then resuspended in an equal volume of 100 mM Tris-HCl (pH 8.0), and hemolysed in 28 volumes of hypotonic buffer [10 mM Tris-HCl (pH 8.0)] at 4 °C. After centrifugation, the ghosts were resuspended in hypotonic buffer, and the procedure was repeated twice in order to obtain hemoglobin-free white ghosts.

Extraction of Lipids from Human Erythrocytes. Human erythrocytes were isolated as described above and hemolysed by addition of an equal volume of H_2O. The hemolysate was extracted with 2-propanol and chloroform according to Rose and Oklander (1965). After evaporation of the solvent, lipids were dissolved in chloroform, and nonsoluble material was removed by centrifugation (3000*g*, 15 min).

Sample Preparation. Lipid or ghost dispersions with or without an appropriate amount of peptide were prepared by one of the following methods: (i) A mixed peptide–lipid film was obtained by drying a peptide–lipid solution (45 μmol of lipid phosphorus) in chloroform/methanol, 1:1 (v/v), on a rotavapor and subsequently stored overnight under high vacuum. This film was hydrated in 1.2 mL of buffer (unless otherwise indicated, the following buffer was used: 150 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4) and was allowed to swell for 3 h at room temperature followed by gentle mixing. (ii) A solution of peptide in DMSO (depending on the final gramicidin concentration of either 1.8 or 18 μmol/mL) was added dropwise (flow rate approximately 40 μL/min) to 60 mL of a vigorously stirred dispersion of ghosts or preformed liposomes (13 μmol of lipid phosphate) in buffer at room temperature. After 30 min of incubation at room temperature, the lipids or ghosts were collected quantitatively by centrifugation (27000*g*, 20 min at 4 °C). In control experiments, DMSO without peptide was added.

Binding Experiments. The actual membrane incorporation of gramicidin or Trp-N-formylated gramicidin was determined by use of the ^{14}C-labeled analogues of the peptides. After addition of 1 mol of peptide per 10 mol of phospholipid by the method described above, the incorporation of ^{14}C label was checked by applying the sample on a linear sucrose gradient from 5% to 20% (w/w) for lipid dispersions or from 20% to 40% (w/w) for ghosts, which were spun at 150000*g* in a Beckman SW 41 rotor at 4 °C for 20 h. In the presence or absence of gramicidin, the ghost membranes as well as the liposomes gave multiple bands at intermediate densities in the gradients. These bands were pooled and taken for ^{14}C and phosphorus measurements according to the method of Rouser et al. (1970). In the absence of membranes, gramicidin and its formylated derivative are pelleted at the bottom of the gradient.

Nuclear Magnetic Resonance (NMR). Proton-noise-decoupled ^{31}P NMR spectra were recorded either on a Bruker WP-200 spectrometer at 81.0 MHz as described by Killian et al. (1985a) or on a Bruker MSL-300 spectrometer at 121.5 MHz as described by Chupin et al. (1987) using an interpulse time of 1.0 s. Spectra were obtained over a temperature range from 4 to 37 °C by accumulating 5000–10 000 free induction decays. Relative amounts of bilayer and H_{II} components present in the spectra were quantified by computer subtraction of spectra of control samples in the absence of peptide (in the case of ghosts only incubated with DMSO), recorded at the same temperature, followed by integration or, with similar results, by area measurements of the estimated pure bilayer

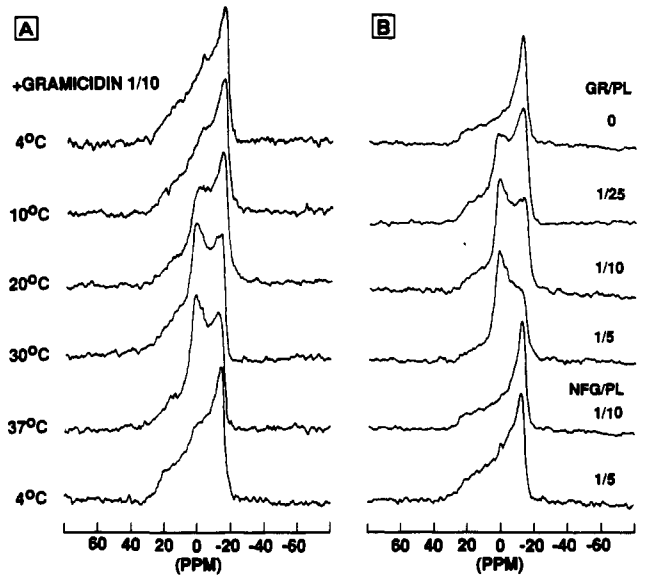

FIGURE 1: 81.0-MHz ^{31}P NMR spectra obtained from aqueous dispersions of (A) erythrocyte lipids in the presence of gramicidin (1:10 mol/mol of phospholipid) recorded at 4, 10, 20, 30, and 37 °C successively after cooling to 4 °C or (B) erythrocyte lipid/gramicidin or erythrocyte lipid/Trp-N-formylated gramicidin mixtures recorded at 37 °C. The molar ratios of peptide to phospholipid are indicated in the figure.

line shape and the H_{II} component in relation to the total area by integration using a Hewlett-Packard digitizer (type 9864 A); 0 ppm corresponds to the chemical shift of the ^{31}P NMR resonance position of lysophosphatidylcholine micelles in water.

X-ray Diffraction (SAXS). Small-angle X-ray diffraction was performed at different temperatures as described previously (Killian et al., 1985a). The exposure time for each measurement was maximally 30 min.

Freeze–Fracture Electron Microscopy (FFEM). For freeze–fracturing, freshly prepared samples were supplemented by 30% (v/v) glycerol as cryoprotectant and equilibrated at the indicated temperature for 30 min prior to quenching by solid/liquid N_2. The frozen samples were subsequently prepared in a Balzers freeze–etch device according to standard procedures. The replicas were examined in a Philips-301 electron microscope.

RESULTS

Gramicidin-Induced Structural Changes in Dispersions of Total Lipid Extract of Erythrocytes. Prior to investigating whether gramicidin is able to modulate the lipid structure in erythrocyte membranes, it was established whether this is the case in model systems prepared from the erythrocyte lipid extract. Figure 1 shows ^{31}P NMR spectra obtained from aqueous dispersions of total lipid extract with and without gramicidin [for a review on the use of ^{31}P NMR, see Cullis and De Kruijff (1979)]. In agreement with earlier observations (Cullis & Grathwohl, 1977; Cullis, 1976), dispersions of the total red cell lipids, in the temperature range from 4 to 37 °C, exhibit a broad anisotropic ^{31}P NMR signal with a high-field peak and a low-field shoulder which is typical for phospholipids organized in a lamellar, liquid-crystalline phase. The upper spectrum in Figure 1B shows the ^{31}P NMR spectrum obtained at 37 °C. The effective chemical shift anisotropy (CSA) of the bilayer signal at this temperature is found to be approximately 37 ppm. Upon lowering the temperature to 4 °C, there is a slight increase in CSA to approximately 39 ppm (data not shown). When gramicidin is incorporated at a 1:10 molar ratio with respect to the phospholipid content, by hydration of a mixed peptide–lipid film, two effects are observed (Figure 1A).

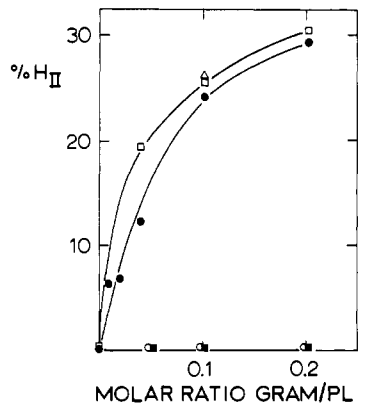

FIGURE 2: Quantification of H_{II} phase formation by gramicidin (●, □) and Trp-N-formylated gramicidin (○, ■) in erythrocyte total lipid extracts (open symbols) and erythrocyte ghosts (closed symbols) at 37 °C. (▲) indicates the amount of H_{II} phase formation induced by gramicidin added externally to erythrocyte lipids.

First, at 37 °C, the ^{31}P NMR spectrum is a superposition of a bilayer component (CSA ~36 ± 1 ppm) and a signal with a reduced CSA of 10 ± 1 ppm and a reversed asymmetry with a low-field peak at approximately +3.5 ppm and a high-field shoulder (as established by subtracting the signal of total lipid extract in the absence of gramicidin at 37 °C). This indicates that at 37 °C a fraction of the lipids is no longer organized in the lamellar phase but adopts a hexagonal H_{II} configuration. Second, several temperature-dependent spectral changes are encountered. Cooling the gramicidin-containing samples from 37 °C leads to a gradual disappearance of the H_{II} component from the ^{31}P NMR spectrum. However, this is paralleled by a broadening of the line shape as compared to the controls without gramidicin, and at 4 °C, the actual line shape of the bilayer type of spectrum of the lipid–gramicidin mixtures is significantly different from that obtained from pure lipid samples. This temperature-dependent behavior is found to be reversible.

Figure 1B shows that at 37 °C the induction of the spectral component indicative of the H_{II} phase is dependent upon the gramicidin to phospholipid ratio. At all ratios tested, the ^{31}P NMR spectra show a reversible disappearance of the H_{II} component upon cooling to below approximately 20 °C, similar to that described for the 1:10 ratio. Moreover, a gramicidin concentration-dependent line broadening is observed at these lower temperatures. The relative amount of phospholipids organized in an H_{II} phase at 37 °C as inferred from ^{31}P NMR data approaches 30% for the highest gramicidin to phospholipid molar ratio (1:5) tested (Figure 2).

Figures 1B and 2 also show that formylation of the four tryptophan residues of gramicidin completely abolishes the induction of the hexagonal H_{II} spectral component by the peptide. Only a line broadening is observed at high peptide to phospholipid molar ratios.

Further information on the structural characteristics of the lipid samples was obtained by small-angle X-ray scattering. Phospholipids organized in multilamellar structures are expected to give rise to Bragg reflections with repeat distances which relate as $1:{}^1/_2:{}^1/_3...$ while a hexagonal H_{II} configuration of lipids will give rise to reflections at repeat distances which relate as $1:(1/\sqrt{3}):{}^1/_2:(1/\sqrt{7})...$ (Luzzati, 1980). The left panel in Figure 3 shows that at 4 °C liposomes of erythrocyte lipids give rise to two reflections with *d* values of 93.8 and 47 Å which relate as $1:{}^1/_2$, consistent with a multilamellar organization. At higher temperatures, the reflections become more broadened, indicating some loss of order in the multilamellar system. In the presence of gramicidin at a 1:10 molar

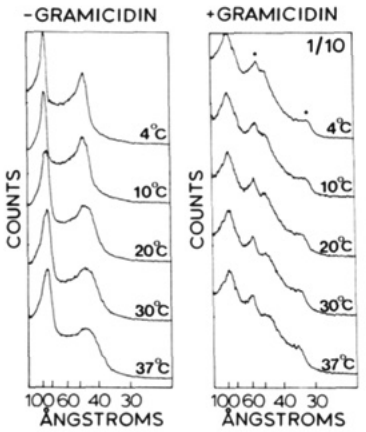

FIGURE 3: Small-angle X-ray diffraction profiles of a dispersion of erythrocyte lipids (left panel) and a mixture of gramicidin and erythrocyte lipids in a molar ratio of 1:10 (right panel) recorded at 4, 10, 20, 30, and 37 °C successively. The H_{II}-specific reflections are indicated with an asterisk.

Table I

molar ratio of peptide to phospholipid	*d* values of reflections (Å) at 4 °C[a] (1)	[1]	(1/2)	[1/√3]	H_{II} tube diameter (Å)
total lipid extract					
no peptide	93.8		47.0		
gramicidin, 1/25	89.2	55.4	45.2	32.6	65.2
gramicidin, 1/10	93.1	54.1	48.3	31.4	62.8
gramicidin, 1/5		53.1		30.3	60.6
NFG, 1/10	89.5		45.9		
ghosts					
no peptide					
gramicidin, 1/10		52.9		30.1	60.2

molar ratio of peptide to phospholipid	*d* values of reflections (Å) at 37 °C[a] (1)	[1]	(1/2)	[1/√3]	H_{II} tube diameter (Å)
total lipid extract					
no peptide	86.0		46.4		
gramicidin, 1/25	84.7	57.3	44.9	33.8	67.6
gramicidin, 1/10	93.1	60.0	47.9	34.7	69.4
gramicidin, 1/5		55.4		31.6	63.2
NFG, 1/10	86.8		44.1		
ghosts					
no peptide					
gramicidin, 1/10		55.7		32.1	64.2

[a] Small angle X-ray diffraction characteristics of erythrocyte lipid, erythrocyte lipid in the presence of gramicidin at various molar ratios, erythrocyte lipids in the presence of Trp-N-formylated gramicidin (NFG), and of ghosts in the presence or absence of gramicidin. Data of recordings at both 4 °C (A) and 37 °C (B) are given. The molar ratios of peptide to phospholipid are indicated in the table. The tube diameters are calculated by multiplying the *d* values of the "$1/\sqrt{3}$" reflections by a factor of 2. The values in brackets indicate the expected relationship between the first and higher order reflections in the lamellar lattice (in parentheses) or hexagonal lattice (in brackets).

ratio (Figure 3, right panel), there are several distinct changes in the diffraction pattern. First, there appears to be a reduction in the total intensity of defined reflections, indicating a loss of long-range order in the system. Second, at all temperatures, two reflections (indicated with an asterisk) are present in addition to those seen in diffraction profiles of the pure lipid system. The *d* values of these reflections relate as $1:(1/\sqrt{3})$ (Table I), thus indicating the presence of an H_{II} phase coexisting with bilayer structures. This is in agreement with the ^{31}P NMR results. However, with respect to the temperature effect, there appears to be a remarkable difference. The X-ray data do not show a significant change in the relative intensity of H_{II} phase specific reflections with decreasing temperatures. Thus, at 4 °C, where the characteristic "H_{II}" type of spectrum

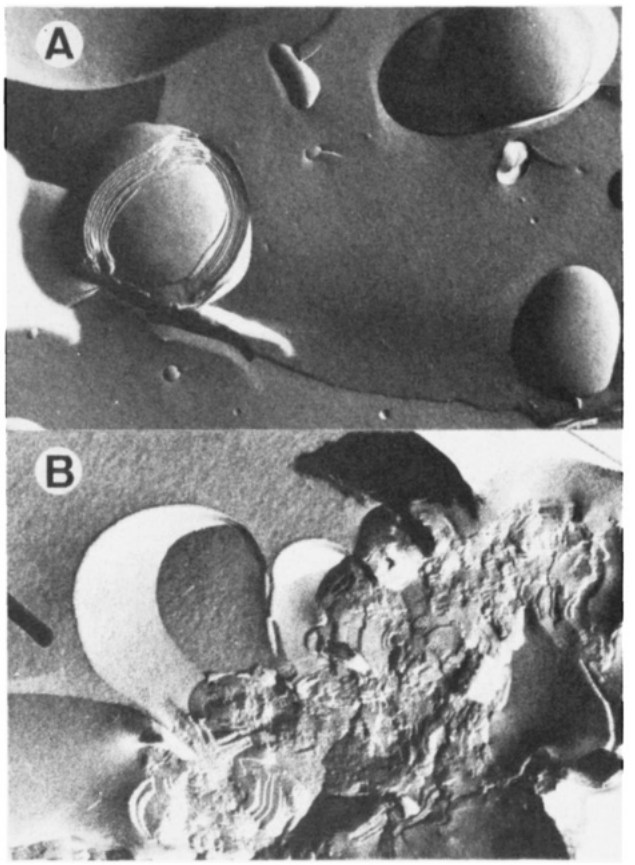

FIGURE 4: Freeze–fracture electron micrographs of dispersions of total lipid extract in the absence (A) (22400×; magnification specified at 8.4-cm column width) or presence (B) (43890×) of gramicidin (1:10 mol/mol of phospholipid), quenched from 37 °C.

is not observed by ^{31}P NMR, the X-ray diffraction data clearly indicate the presence of this phase.

From the *d* values which can be calculated from the "$1/\sqrt{3}$" reflections, it can be derived that there is a slight increase in tube diameter with temperature ranging from 62.8 Å at 4 °C to 69.4 Å at 37 °C (Figure 3 and Table I). This is in marked contrast to the temperature-dependent decrease in tube diameter (which reflects the decrease in effective length of the acyl chains at higher temperatures) usually encountered in pure lipid systems (Seddon et al., 1984) and the temperature-independent tube diameter of DOPC in the H_{II} phase with gramicidin (Killian et al., 1985a; Chupin et al., 1987). When gramicidin is incorporated at different peptide to lipid ratios, the following results are obtained. The two additional reflections arising from the H_{II} phase are already detectable at a 1:25 gramicidin to phospholipid molar ratio. The intensity of these reflections increases with increasing gramicidin to lipid ratios (not shown) whereas the tube diameter decreases slightly with increasing gramicidin to phospholipid ratios (Table I). Addition of Trp-N-formylated gramicidin to the total lipid extract in ratios up to 1:5 (moles per mole of phospholipid), does not lead to any change in the diffraction profile. At all temperatures, two reflections are observed very similar to that observed for the multilamellar peptide-free liposomes (data not shown).

A third technique which has been applied to evaluate gramicidin-induced changes in model membrane morphology is freeze–fracture electron microscopy (Verkleij, 1984). While pure lipids form extended multilamellar structures with smooth fracture faces at 0 °C (not shown) and 37 °C (Figure 4A), incorporation of gramicidin in a 1:10 molar ratio to phospholipid leads to a drastic change in morphology (Figure 4B).

Table II: Recovery (%) of Added Peptide Associated with Membranes[a]

molar ratio of peptide to phospholipid	total lipid extract		ghosts externally added
	mixed film	externally added	
gramicidin, 1:10	95	88	97
NFG, 1:10	96	90	>50

[a] Incorporation of gramicidin and Trp-N-formylated gramicidin (NFG) in total lipid extracts and ghosts was determined by gradient centrifugation. For experimental details, see Materials and Methods.

Besides residual bilayer structures, short, highly curved hexagonal H_{II} tubes characterized by striated fracture faces are observed. There are virtually no differences observed in the lipid structure of samples quenched from 0 °C (not shown) and 37 °C (Figure 4B).

In studies on the effects of gramicidin or its formylated analogue on erythrocyte ghosts, to be presented below, the peptides have to be added externally to the membranes. We therefore compared the H_{II} phase inducing ability of gramicidin when added externally from dimethyl sulfoxide to preformed liposomes with that observed when gramicidin is incorporated by the mixed film method. A typical result for the quantification of H_{II} phase formation by externally added gramicidin is included in Figure 2. Very similar results were obtained for both methods as revealed by ^{31}P NMR and SAXS (data not shown).

The actual incorporation of gramicidin or Trp-N-formylated gramicidin was determined by using ^{14}C-labeled analogues and sucrose gradient centrifugation. The major part (approximately 90%) of the added peptide for both gramicidin and Trp-N-formylated gramicidin was isolated associated with lipid (Table II).

Gramicidin-Induced Modulation of Lipid Structure in Erythrocyte Ghosts. To study the ability of gramicidin to induce a hexagonal H_{II} phase in the erythrocyte membrane, we performed the same series of experiments as described for total erythrocyte lipids but now using erythrocyte ghosts and incorporating peptides by external addition from DMSO. The extent of incorporation of gramicidin in ghosts was found to exceed 90% (Table II). Only approximately 50% of the formylated gramicidin was isolated from the gradient associated with the membranes. The remainder of this peptide was found in a pellet at the bottom of the gradient together with part of the membrane lipids.

The ^{31}P NMR spectra obtained from ghost suspensions at 37 °C (Figure 5B, upper spectrum) and at all temperatures tested (data not shown), in agreement with earlier findings (Cullis & Grathwohl, 1977), indicate a liquid-crystalline bilayer organization of the phospholipids. Addition of excess DMSO (3%, v/v), which is 3 times higher than the highest concentration introduced by the addition of gramicidin, did not lead to any detectable change in lipid structure (^{31}P NMR, data not shown). The magnitude of the CSA, ~39 ppm at 4 °C and ~37 ppm at 37 °C, is comparable to that found in spectra from dispersions of total lipid extract. As shown in Figure 5A, at 37 °C the incorporation of gramicidin in a 1:10 molar ratio in erythrocyte ghosts leads to a similar induction of an "H_{II}" type of ^{31}P NMR spectral component as observed in the derived liposomes (Figure 1). The CSA of this component at 37 °C is 10 ± 1 ppm while the residual chemical shift anisotropy of the bilayer component at this temperature is approximately 35 ppm. This means that incorporation of gramicidin, next to structural changes, causes a reduction of the CSA from 37 to 35 ppm at 37 °C for phospholipids in the residual bilayer. The gramicidin-dependent induction of the

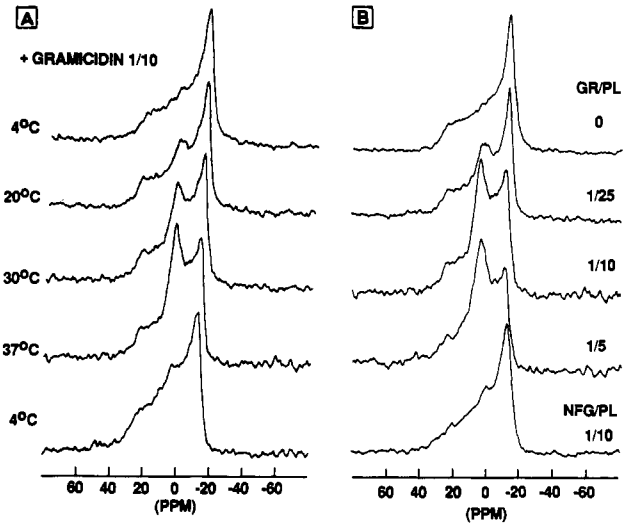

FIGURE 5: 121.5-MHz ^{31}P NMR spectra obtained from aqueous dispersions of erythrocyte ghosts (A) after external addition of gramicidin (1:10 mol/mol of phospholipid) recorded at 4, 20, 30, and 37 °C successively and finally after cooling to 4 °C or (B) after external addition of various amounts of gramicidin or its formylated derivative recorded at 37 °C. The numbers refer to the peptide to phospholipid molar ratios.

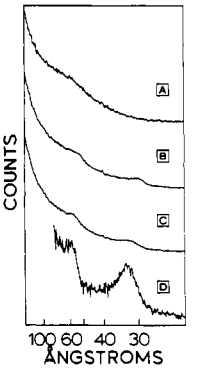

FIGURE 6: Small-angle X-ray diffraction patterns of ghosts at 37 °C (A) and ghosts in the presence of gramicidin (1:10 mol/mol of phospholipid) at 4 (B) and 37 °C (C). (D) is obtained by subtraction of (A) from (C).

"H_{II}" spectral component in ghosts (Figure 5A) shows a similar temperature-dependent behavior as is seen in the total lipid extract (Figure 1). Lowering the temperature from 37 to 4 °C causes a gradual disappearance of the H_{II} component from the ^{31}P NMR spectrum which is accompanied by a broadening of the spectrum although less pronounced than that seen with total lipid extracts. The CSA of the bilayer component is slightly increased from 35 ppm at 37 °C to 37.5 ppm at 4 °C.

Figure 5B shows the gramicidin concentration dependence of H_{II} phase induction as revealed by ^{31}P NMR spectroscopy at 37 °C. Quantitation of these data shows that the molecular efficiency of the H_{II} phase formation by gramicidin is only slightly less in ghost preparations than that in total lipid dispersions (Figure 2). In the range of a 1:80 to 1:50 molar ratio of gramicidin to phospholipid, a H_{II} signal can be detected. In line with the observation with the total lipid dispersions, incorporation of Trp-N-formylated gramicidin does not lead to the induction of an H_{II} type of ^{31}P NMR spectral component. Only a slight change in line shape is observed.

SAXS profiles of ghost preparations with and without gramicidin are shown in Figure 6. For ghosts in the absence of gramicidin, only a random scattering profile is observed (Figure 6A), indicating a lack of long-range order in the suspension of the erythrocyte membranes. Upon addition of gramicidin in a 1:10 molar ratio with respect to the phos-

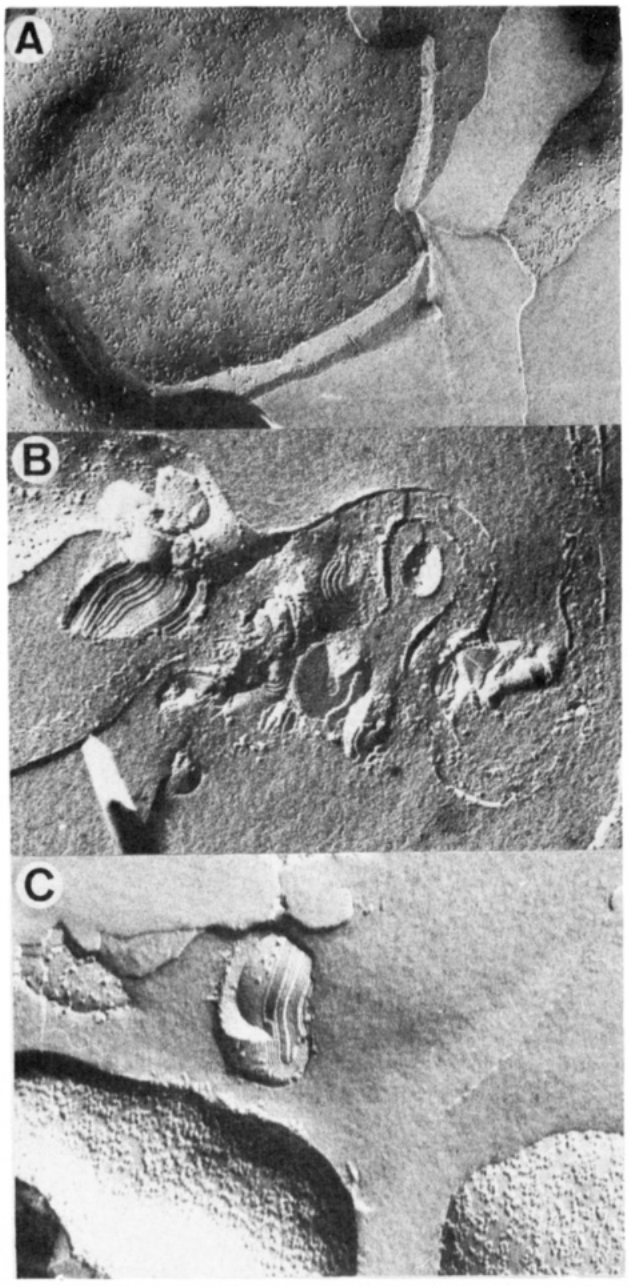

FIGURE 7: Freeze–fracture electron micrographs of ghost dispersions in the absence of gramicidin quenched from 37 °C (A) and in the presence of gramicidin at a molar ratio of 1:10 mol/mol with respect to phospholipid quenched from 0 °C (B) and 37 °C (C) (43890×).

pholipids, two small reflections arise at both 4 °C (B) and 37 °C (C). When the random scattering profile is subtracted from these signals (Figure 6D), two reflections are obtained with d values which relate as $1:(1/\sqrt{3})$ (Table I). This indicates the existence of lipids organized in a hexagonal H_{II} configuration. The X-ray data also display the unusual temperature-dependent increase in tube diameter of the gramicidin-induced H_{II} phase from approximately 60 Å at 4 °C to approximately 64 Å at 37 °C.

As it can be concluded that gramicidin is also capable of inducing hexagonal H_{II} structures in erythrocyte ghosts, it is of considerable interest to obtain more insight in the actual localization of these structures within the samples. Freeze–fracture electron micrographs are shown in Figure 7. In erythrocyte ghost preparations treated with or without 1% (v/v) DMSO, extended bilayers can be observed. The smooth fracture faces of the ghost membrane are covered with proteinaceous intramembranous particles which are slightly aggregated as is generally observed in ghost preparations. In contrast, freeze–fracture replicas of gramicidin-containing ghosts clearly show highly curved and short hexagonally organized tubes which are associated with the membranes as can be inferred from the presence of the intramembranous particles next to the H_{II} tubes. These tubes do not contain particles. There is no appreciable difference observed between samples quenched from 4 °C (Figure 7B) and 37 °C (Figure 7C).

Discussion

From the combined results from ^{31}P NMR, SAXS, and FFEM techniques, it can be concluded that at 37 °C gramicidin is capable of inducing hexagonal H_{II} structures not only in human erythrocyte total lipid extracts but also in ghosts. This is the first example in which peptide-induced H_{II} phase formation is demonstrated in a biomembrane, and in conjunction with the preceding paper (Classen et al., 1987), the results of this study allow us to obtain more insight in the possible mechanisms of peptide-induced transbilayer reorientation of phospholipids.

It is clearly shown that the induction of H_{II} phase formation in total lipid extract as well as in ghosts is dependent on the gramicidin concentration. At ratios of gramicidin to membrane phospholipid exceeding 1:80 to 1:50 (mole per mole) in ghosts at 37 °C, an H_{II} configuration can be detected by ^{31}P NMR. This onset of gramicidin-induced H_{II} phase formation occurs at much lower concentrations than in DOPC model membranes in which H_{II} phase formation was only detected at concentrations of gramicidin exceeding 1:15 mol/mol of phospholipid (Killian & De Kruijff, 1985a). This difference may be due to the presence of lipid species in the membrane which as such favor packing in H_{II} phases (e.g., phosphatidylethanolamines or long-chain lipids) or cause an increased tendency of gramicidin to form lateral aggregates which is found to be a prerequisite for H_{II} phase formation (Killian et al., 1986, 1987; Chupin et al., 1987). From quantification of the ^{31}P NMR data, the amount of H_{II} phase can be calculated as a function of the gramicidin content. From the initial slopes of the curves in Figure 2, at gramicidin:phospholipid ratios up to 1:25, stoichiometries for H_{II} phase formation of about 5 and 3 mol of phospholipid/mol of gramicidin can be inferred for lipid extracts and ghosts, respectively. The difference is most likely due to the presence of proteins in the ghost membranes which influence the gramicidin–lipid interaction. At higher gramicidin to phospholipid molar ratios (>1:10), the efficiency to induce H_{II} phase formation is reduced in both systems as can be seen from the decrease in slopes. The molecular efficiency of H_{II} phase formation in these systems is less than the stoichiometry of 7 mol/mol found for a gramicidin-induced H_{II} phase in DOPC model systems (Killian et al., 1987; Chupin et al., 1987). However, we have to keep in mind that in erythrocyte total lipid extract and ghosts, approximately equal amounts of cholesterol and phospholipids are present. Cholesterol is possibly also transferred to the H_{II} phase (but is not detected by ^{31}P NMR), thereby seemingly reducing the phospholipid to gramicidin stoichiometry. The H_{II} signal induced by gramicidin exhibits a very small CSA; the low-field peak is detected at approximately +3.5 ppm, and the CSA measured from the signal is 10 ± 1 ppm. It has to be emphasized that the signal is not due to isotropic movement of phospholipids but has a clearly asymmetrical line shape. The residual CSA is smaller than that expected and observed in pure lipid systems (Cullis & De Kruijff, 1978; Cullis, 1980) when only long-axis rotation and lateral diffusion around the H_{II} tubes average the ^{31}P NMR chemical shift tensors (Cullis & De Kruijff, 1979;

Seelig, 1978). As suggested previously (Van Echteld et al., 1982), for gramicidin-induced H_{II} phase formation in phosphatidylcholine systems this is most likely due to a decrease in head-group order at the level of the phosphate region, caused by a high gramicidin content in the H_{II} phase which leads to a spacing of the phospholipids. Furthermore, while individual phospholipid species exhibit slightly different CSA's (Seelig, 1978; Cullis & De Kruijff, 1978) and the phospholipid composition of the H_{II} phase is as yet unknown, the CSA of the H_{II} phase need not be related to that of the bilayer phase. The lipid composition of the H_{II} phase and possibly preferential interactions in the lamellar phase will be the subject of further investigations. In this respect, the occurrence of multiple bands obtained with sucrose density centrifugation of ghosts after the addition of gramicidin, which are indicative for phase separation (Killian et al., 1987), offers a promising future approach toward these questions.

The lipid-modulating ability of gramicidin is specific and is clearly determined by the chemical structure of the peptide in that Trp-N-formylated gramicidin lacks this property as observed by ^{31}P NMR and SAXS. This inability to induce the H_{II} phase is not due to the low extent of incorporation (Table II). As has been shown by Killian et al. (1985), the tryptophans of the gramicidin molecule are essential for the H_{II}-inducing ability in DOPC model membranes. Modification by formylation of the indolamides leads to an abolishing of this capacity, probably as a result of different aggregational behavior of the modified peptide in combinaion with a different monomer conformation (Killian et al., 1987; Killian & De Kruijff, 1987).

The diameter of the H_{II} phase tubes at 37 °C is approximately 64 Å in ghosts and approximately 70 Å in total lipid extracts, which is slightly smaller than the tube diameters found by Killian et al. (1986) for the gramicidin-induced H_{II} phase in DOPC (72.4 Å) and other lipid systems (70–72 Å). The H_{II} phase formation in erythrocyte-derived systems exhibits several interesting temperature-dependent features. First, a temperature-dependent increase in tube diameter was observed in total lipid extracts as well as in ghosts which contrasts the usually observed decrease in tube diameter with increasing temperature compatible with a reduction in effective chain length upon increasing temperature (Seddon et al., 1984). Preliminary results show that in DOPC–cholesterol–gramicidin model systems, there is a similar temperature-dependent increase in tube diameter (M. Gasset, unpublished observations). This indicates that cholesterol or a cholesterol–gramicidin interaction could be responsible for this unusual temperature dependence of the tube diameter. Second, there is an apparent discrepancy between data obtained at lower temperatures with ^{31}P NMR and that obtained with SAXS and FFEM. In erythrocyte-derived systems in the presence of gramicidin at 0–4 °C, SAXS and FFEM results indicate, in contrast to the ^{31}P NMR data, the presence of lipids organized in a hexagonal H_{II} phase. Because under our conditions neither the SAXS not the FFEM technique necessarily gives quantitative information on the H_{II} phase present, the exact amount of this lipid phase at these temperatures is unknown. However, the results clearly show that H_{II} phase formation also occurs at 4 °C. In this respect, it is relevant to note that it has been described that the lateral diffusion of phospholipids in human erythrocyte membranes varies (linearly) approximately 50-fold between 42 and 1 °C (Bloom et al., 1983). When we assume the same temperature dependence of lateral diffusion of erythrocyte phospholipids around the H_{II} phase tubes in the presence of gramicidin, the correlation time (τ_c) of this movement also varies 50-fold in this temperature range. According to Burnell et al. (1980), τ_c is related to the diffusion coefficient by

$$1/\tau_c = (6/r^2)(D_{diff} + D_t)$$

where τ_c = the correlation time for isotropic motion, r = the radius of the H_{II} tubes, D_{diff} = the lateral diffusion coefficient, and D_t = the tumbling-dependent part of the Brownian rotational diffusion, which we assume to be 0. Using this equation and the D_{diff} data derived from Bloom et al. (1983), we can calculate that at 37 and 4 °C $\tau_c = 9 \times 10^{-7}$ and 2×10^{-5} s, respectively (taking into account the increased radius of the H_{II} phase tubes with temperature). At 4 °C, the τ_c exceeds 10^{-5} s, which may lead to an incomplete motional averaging of the chemical shift tensors (Burnell et al., 1983). Moreover, in the gramicidin-rich H_{II} cylinders, restriction of the lateral diffusion of lipid molecules might occur due to the presence of (ordered) gramicidin aggregates. Tank et al. (1982) showed that high concentrations of the polypeptide restrict the lateral mobility of membrane components. Thus, it is quite possible that below 20 °C only a broadened "bilayer" ^{31}P NMR line shape is observed from lipids organized in cylindrical structures. These results once again emphasize the need for a multiple-technique approach to monitor lipid structures as has been indicated before by Hui et al. (1980). From the present data, we therefore cannot conclude that there is an increase in H_{II} phase formation with temperature as suggested by the ^{31}P NMR data.

Notwithstanding the occurrence of highly unsaturated phosphatidylethanolamines, the lipids in the erythrocyte membrane experience a very stable bilayer organization (Cullis & Grathwohl, 1977) which most likely is the result of its lipid composition in possible conjunction with the bilayer-stabilizing action of integral membrane proteins such as glycophorin (Taraschi et al., 1982a,b). Even proteolytic (Cullis & Grathwohl, 1977) and phospholipase treatments (Van Meer et al., 1980) do not result in a loss of bilayer structure of the membrane phospholipids. Only very large amounts of fusogens as glyceromonooleate (>1 mol/mol of phospholipid) have been shown to lead to an H_{II} phase formation in intact erythrocytes (Cullis & Hope, 1978; Hope & Cullis, 1981). Considering the relatively small amounts of gramicidin which induce H_{II} phase formation, it can be concluded that this peptide is an extremely potent modulator of membrane lipid structure. The FFEM experiments on ghosts show that the intramembranous particles representing the intrinsic membrane proteins are in close association with the H_{II} structures. We thus can conclude that the H_{II} phase originates from the membrane and is not the result of extraction of lipids by DMSO which is added to introduce the gramicidin.

In pure lipid model membranes, a relationship exists between the tendency to form nonbilayer structures and the increase of transbilayer movement of phospholipid (Gerritsen et al., 1980; Noordam et al., 1981). In line with these observations, the ability of gramicidin to induce H_{II} phase formation in erythocyte membranes as studied in this paper and the ability to induce an increase in flip rate of lysopalmitoylphosphatidylcholine and palmitoylcarnitine in erythrocytes as shown in the preceding paper (Classen et al., 1987) suggest that these phenomena are related. This is strongly supported by the finding that modification of gramicidin by formylation of the four tryptophan residues abolishes not only the ability to induce an H_{II} phase but also the ability to promote transbilayer movement of lipids and to induce a nonspecific permeability (Classen et al., 1987). On the other hand, it is noteworthy that increased transbilayer movement already

starts at a molar ratio of 1:2000 whereas H_{II} phase formation can be detected only at ratios exceeding 1:80 moles of gramicidin per mole of phospholipid. This indicates that increased lipid transbilayer movement starts before H_{II} phase formation.

Summarizing, the action of gramicidin on the erythrocyte membrane can be described as follows. At very low concentrations, only specific permeation occurs through gramicidin channels, which is apparent from K^+ efflux data presented in the preceding paper (Classen et al., 1987). Channel formation probably occurs by dimerization due to N-terminal to N-terminal association of gramicidin (Urry, 1971; Wallace, 1981). Starting at a molar ratio of peptide to phospholipid of 1:2000, an enhancement of flip rate becomes detectable, and at approximately the same molar ratio, a nonspecific (nonchannel) permeability to ions (choline and oxalate) and nonelectrolytes (erythritol and sucrose) is observed. Only at much higher ratios (>1:80) does H_{II} phase formation become detectable. It thus appears that the processes leading to the onset of H_{II} phase formation rather than this phase itself are responsible for flip enhancement and nonchannel permeability. Since channel formation as such does not cause flip enhancement as discussed in the preceding paper (Classen et al., 1987), aggregate formation by gramicidin, which has been proposed to precede H_{II} phase formation, should be considered. Such gramicidin aggregate structures have been observed at low water content and cause a decrease in acyl chain order in DOPC model membranes (Chupin et al., 1987; Killian & De Kruijff, 1985b). This decrease in order in the lipid phase together with the specific structure and topology of these aggregates might cause an enhancement of transbilayer movement due to reorientation of lipids from their position at the lipid–water interface to a more favorable localization in these intermediate structures, thereby reducing the energy of activation of transbilayer movement. A detailed description of the molecular mechanism of gramicidin-induced H_{II} phase formation will be published elsewhere (Killian & De Kruijff, 1987). Since gramicidin aggregate formation precedes H_{II} phase formation, this process can occur at much lower concentrations of gramicidin in the membrane than is necessary for H_{II} phase formation. Furthermore, in this concept, the observed lag phase in flip enhancement at lower gramicidin concentrations, as discussed in the preceding paper (Classen et al., 1987), might be due to the time required to obtain these gramicidin aggregates. Aggregate formation can be expected to be determined by, e.g., lateral diffusion of the peptide in the lipid phase, the temperature, and the gramicidin concentration.

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Solid-State ^{15}N NMR of Oriented Lipid Bilayer Bound Gramicidin A′ †

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ABSTRACT: Highly oriented samples of lipid and gramicidin A′ (8:1 molar ratio) have been prepared with the samples extensively hydrated (approximately 70% water v/w). These preparations have been shown to be completely in a bilayer phase with a transition temperature of 28 °C, and evidence is presented indicating that the gramicidin is in the channel conformation. An estimate of the disorder in the alignment of the bilayers parallel with the glass plates used to align the bilayers can be made from the asymmetry of the nuclear magnetic resonances (NMR). Such an analysis indicates a maximal range of disorder of ±3°. Uniformly ^{15}N-labeled gramicidin has been biosynthesized by *Bacillus brevis* grown in a media containing ^{15}N-labeled *Escherichia coli* cells as the only nitrogen source. When prepared with labeled gramicidin, the oriented samples result in high-resolution ^{15}N NMR spectra showing 12 resonances for the 20 nitrogen sites of the polypeptide. The frequency of the three major multiple resonance peaks has been interpreted to yield the approximate orientation of the N–H bonds in the peptide linkages with respect to the magnetic field. These bond orientations are only partially consistent with the extant structural models of gramicidin.

The spectroscopic study of lipid bilayer bound proteins and polypeptides has been severely hampered by the large size of the aggregated system. A variety of techniques have been employed to circumvent the resultant problems of long correlation times and light scattering. Chemical modifiers (e.g., lysolipids or detergents) or mechanical modifiers (e.g., sonication) of these samples alter the nature of the preparation. The consequences of such sample modifications for the conformational and dynamic properties of the protein or polypeptide are significant. Solid-state nuclear magnetic resonance (NMR)[1] spectroscopy is an approach that requires the very long correlation times associated with extensive lipid bilayers. Here, the pentadecapeptide gramicidin A′ has been isotopically labeled with ^{15}N, incorporated into bilayers of dimyristoylphosphatidylcholine (DMPC), hydrated, and oriented such that the lipid bilayers are uniformly parallel with each other, and then studied by ^{15}N and ^{31}P solid-state NMR.

Gramicidin A′ is a linear polypeptide produced by *Bacillus brevis* during the early stages of its sporulation cycle. In lipid bilayers, it forms a monovalent cation-selective channel that has been extensively studied. The channel is formed by a dimer of gramicidin, and in single-channel conductance studies, the lifetime of the dimer has been measured. The lifetime is highly dependent on the length of the fatty acyl chains of the lipids; in DMPC, the hydrophobic width of the bilayer coincides with the length of the gramicidin dimer, and long lifetimes are expected (Wallace et al., 1981). A model of the channel, which was developed by means of conformational analysis, has been extant since 1971 (Urry, 1971). However, confirmation by an atomic resolution structure determination has not been achieved for gramicidin in the presence of lipids, because of the difficulty of forming cocrystals of the polypeptide and lipid which diffract to high resolution (Wallace, 1986). Urry's model, which is generally accepted as an approximate description of how the polypeptide backbone is folded, predicts in the rigid limit a structurally uniform pore that may not completely account for cation binding sites (Urry et al., 1982) and potential energy barriers to cation passage (Eisenman & Sandblom, 1984).

Not only is a higher resolution structure determination of interest but also the dynamics of this molecule appear to be very interesting. It is thought that the peptide linkages that line the channel pore rotate so that the carbonyl oxygens can coordinate the cation during its passage across the membrane. While there have been several efforts to calculate the local motions of the peptide linkages (Fischer & Brickmann, 1983; Venkatachalam & Urry, 1984; MacKay et al., 1984), it has not been experimentally characterized. Consequently, gramicidin A still poses a number of interesting structural and dynamic questions.

Recently, the question of gramicidin dynamics has been approached with both ^{13}C (Smith & Cornell, 1986) and ^{2}H

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; DSC, differential scanning calorimetry; DMPC, dimyristoylphosphatidylcholine; CSA, chemical shift anisotropy; ppm, parts per million; MSA, molecular symmetry axis.

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