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Gramicidin Conformational Studies with Mixed-Chain Unsaturated Phospholipid Bilayer Systems†

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ABSTRACT: The transition of gramicidin from a nonchannel to a channel form was investigated using mixed-chain phosphatidylcholine lipid bilayers. Gramicidin and phospholipids were codispersed, after removal of the solvents chloroform/methanol or trifluoroethanol which resulted in nonchannel and channel conformations, respectively, as confirmed using circular dichroism (CD). The fluorescence emission maxima of the nonchannel form were shifted toward shorter wavelengths by heating at 60 °C (for 0–12 h), which converted it to a channel form, again as confirmed by CD. The channel form did not respond to heat treatment. Heat treatment also increased the fluorescence anisotropy of the nonchannel gramicidin tryptophans. The rate of transition from the nonchannel to channel conformation was found to be faster if phosphatidylethanolamine was present in combination with phosphatidylcholine compared to phosphatidylcholine alone. Also, gramicidin in bilayers of the polyunsaturated 1-palmitoyl-2-docosahexaenoylphosphatidylcholine converted more rapidly compared to 1-palmitoyl-2-oleoylphosphatidylcholine. Using the fluorescence anisotropy of the membrane lipid probe 1,6-diphenyl-1,3,5-hexatriene, it was also shown that the motional properties of the surrounding lipid acyl chains differed for the channel and nonchannel gramicidin conformations. The possibility that lipids tending to favor the hexagonal phase (H_{II}) would enhance the rate of the nonchannel to channel transition was supported by ^{31}P NMR which revealed the presence of some H_{II} lipids in the channel preparations. The results of this study suggest that gramicidin may serve as a useful model for similar conformational transitions in other more complex membrane proteins.

Gramicidin is a hydrophobic pentadecapeptide composed of alternating L- and D-amino acids which span the lipid bilayer to form a cation channel in the form of a β6.3 left-handed helical dimer linked at the N-terminals [see reviews by Anderson (1984), Urry (1985), Cornell (1987), and Wallace (1990)]. The structure is stabilized by alternate intra- and intermonomer hydrogen bonds from the amide NH and formyl C=O groups. One motivation for the study of gramicidin is that is can be used as a model to help in the understanding of more complex systems. This applies not only to its function as an ion channel but also as a model for protein/lipid interactions in general.

Gramicidin has two distinct conformational states which were first recognized in organic solvents [see recent review by Wallace et al. (1990)]. In trifluoroethanol and DMSO, gramicidin is in a monomeric β6.3-helical form. By contrast, in ethanol or chloroform/methanol, a double-helical dimer configuration is found. Using circular dichroism (CD)[1] and NMR, it has recently been shown that when gramicidin is added to lipid bilayers, from trifluoroethanol or DMSO, the β6.3 single helical (channel) conformation is maintained in the bilayer, with two monomers joined at the N-termini, at the bilayer center, to form a dimer comprising the channel configuration (LoGrasso et al., 1988; Killian & Urry, 1988; Killian et al., 1988a,b). A nonchannel (double-helical head-to-tail dimer) conformation resulted if the protein was added from a solution of ethanol or chloroform/methanol. The solvent dependence occurs even if the solvent is completely removed prior to dispersing. The solvent-directed conformation in lipid bilayers has also been demonstrated using high-pressure liquid chromatography where the two forms of gramicidin were recovered from lipid bilayers in their monomeric (channel) and dimeric (helical) configurations (Bano et al., 1989, 1991). A solvent-dependent formation of H_{II} lipids with DOPC has also been demonstrated, with the addition of gramicidin (from trifluoroethanol or DMSO) to preformed bilayers inducing the formation of H_{II} lipids, while addition from ethanol maintained the bilayer phase (Tournois et al., 1987). In earlier studies, it was shown that to obtain gramicidin in a channel form it was necessary to heat phospholipid and gramicidin dispersions at elevated temperatures for several hours [see, for

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[1] Abbreviations: CD, circular dichroism; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; H_{II}, hexagonal phase; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; PC, phosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoyl-PC; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-PC; POPE, 1-palmitoyl-2-oleoyl-PE.

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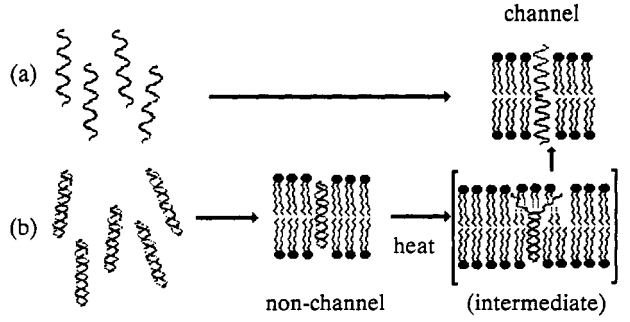

FIGURE 1: Diagram illustrating the incorporation of gramicidin into lipid bilayers (gramicidin:phospholipid ratio 1:10) from (a) trifluoroethanol, resulting in the channel form, or (b) ethanol or chloroform/methanol, resulting in the nonchannel form. Prolonged heating converts the nonchannel to the channel form.

example, Urry et al. (1979) and Spinsi et al. (1979)]. In light of more recent work (LoGrasso et al., 1988; Killian & Urry, 1988; Killian et al., 1988a,b; Bano et al., 1989, 1991), it has become clear that in the above studies a nonchannel form was initially formed which was then converted into the channel form by the heating process, as illustrated in Figure 1.

There are four tryptophan side chains in the C-terminal half of the molecule which protrude into, and potentially interact with, the adjacent lipid acyl chains. It has been shown that the tryptophans are essential for the cation channel to function properly, by substitution with other amino acids (Heitz et al., 1982; Prasad et al., 1983; Trudelle & Heitz, 1987), or after photolysis (Jones et al., 1986; Busath & Hayon, 1988). The fluorescence emission spectra of gramicidin have been shown to be sensitive to both the solvent and lipid environment (Masotti et al., 1986). Time-resolved fluorescence decay studies indicated a complex decay with three components. The motional properties of the tryptophans have also been investigated, and it was noted that the lipid phase may affect this property (Scarlata, 1988). In spite of the studies on the fluorescent properties of gramicidin, the relationship of the channel and nonchannel conformations and the heat-induced interconversion with the fluorescence properties needs to be clarified. This was one of the purposes of the present study. Further, the use of these fluorescent properties to determine the lipid dependence of the conversion from nonchannel to channel conformations was explored. It was shown that the different conformations could be distinguished on the basis of the fluorescence emission maxima, the tryptophan fluorescence anisotropy, and also by the fluorescence anisotropy of adjacent lipid bilayer probes such as DPH. It was also found that PE and cis unsaturation enhance the heat-induced nonchannel to channel conversion.

Experimental Procedures

Materials

Gramicidin D was obtained from Sigma (St. Louis, MO) and used without further purification. Lipids were obtained from Avanti Lipids (Birmingham, AL). DPH was from Molecular Probes (Eugene, OR). The solvents chloroform and methanol (HPLC grade) were from Fisher Scientific (Pittsburgh, PA), trifluorethanol was from Aldrich (99%+, Milwaukee, WI), and ethanol (200 proof, anhydrous) was from Warner–Graham (Cockeysville, MA).

Methods

Preparation of Lipid Vesicles. Vesicles containing gramicidin were made as described elsewhere (LoGrasso et al., 1988). Briefly, lipids in chloroform were first dried down under a stream of nitrogen to form a thin film in a test tube. Gramicidin was then added from a trifluoroethanol or chloroform/methanol (97:3 by volume) solution, and the solvent was again removed by nitrogen. The film of lipid/gramicidin was kept thin to prevent trapped solvent so that prolonged evacuation was not necessary, although this was used on some of the earlier preparations. In the cases where DPH was used, a solution of DPH in tetrahydrofuran was added and the solvent removed before the addition of gramicidin. Buffer (10 mM Tris-HCl/150 mM NaCl, pH 7.4) was then added, and the contents of the tube were warmed to ~37 °C. The contents were then subjected to sonication for a total of 4 min at 4 °C. Due to the gramicidin presence, the solution did not clarify, and the vesicles obtained were of a heterogeneous size. The vesicles were then subjected to centrifugation at 1100*g* for 5 min to remove large particles. Following this, the vesicles were diluted 10-fold to a working concentration of 50 μM for the fluorescence, or 500 μM for the CD measurements.

The amount of incorporation of the gramicidin into the vesicles was verified by measurement of the lipid phosphorus (Bartlett, 1959) and of the gramicidin by absorption at 280 nm and using a standard curve (in methanol). The ratio stated under Results refers to moles of gramicidin to moles of phospholipid in the vesicles.

The preparations of gramicidin/PDPC were checked for oxidation by TLC of a chloroform/methanol lipid extract (Bligh & Dyer, 1959) prepared from the vesicle preparation (after heat treatments of up to 12 h at 60 °C). TLC failed to reveal trailing spots, diagnostic of oxidized lipids. There was also an absence of conjugated dienes, indicative of oxidation (Klein, 1970). Finally, the extracted PDPC was saponified, and the fatty acid methyl esters were obtained and examined by GLC. A loss of the docosahexaenoate would have resulted in a greater proportion of palmitate being indicated since palmitate is much less susceptible to oxidation; however, the stoichiometry of the palmitate on the *sn*-1 position and of the docosahexaenoic acid on the *sn*-2 position of the phosphatidylcholine was found to remain at 1:1.

CD Spectra. CD spectra were obtained on a AVIV 60DS spectropolarimeter with computer-controlled data acquisition and analysis. Samples were prepared as for fluorescence measurements except that the lipid concentration was 500 μM. The path length was 1 cm, and vesicles without gramicidin were taken as the base line. With the sonicated vesicles, some scattering was observed at lower wavelengths; however, this did not affect the ability of the spectra to distinguish between channel and nonchannel conformations.

Fluorescence Measurements. Emission spectra (uncorrected) were obtained using an SLM 48000 spectrofluorometer using a Neslab refrigerated water bath to maintain temperature (monitored using a Jenco digital thermometer). Fluorescence anisotropy measurements were made as previously described (Stubbs et al., 1984). For measurement of the anisotropy of the tryptophans of gramicidin, excitation was at 280 nm, and emission was observed through a 10-nm band-pass interference filter at 340 nm. For DPH, excitation was at 360 nm with a 10-nm band-pass interference filter at 430 nm. In order to avoid light-scattering effects on the fluorescence anisotropy, the vesicles were diluted until this effect was minimized. This was achieved by plotting the fluorescence anisotropy with the optical density of successively diluted solutions. It was found that at 50 μM scattering effects on the fluorescence anisotropy were negligible and this concentration was used for subsequent experiments.

The fluorescence decay of the gramicidin tryptophans was determined using a Coherent (CR-12) argon ion laser pumped

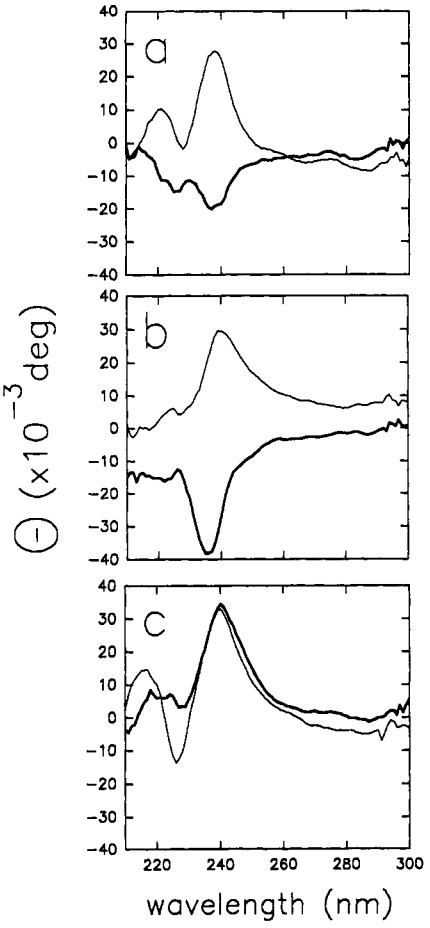

FIGURE 2: CD spectra of gramicidin in POPC vesicles (500 μM lipid, 25 °C, gramicidin:phospholipid ratio 1:10) using the following solvents (removed prior to codispersing): (a) chloroform/methanol (97:3 by volume); (b) ethanol; (c) trifluoroethanol. The thin lines are for vesicles which were heated at 60 °C for 12 h prior to measurement. The thick lines are for samples which were kept at 4 °C for 12 h. Spectra were collected at 25 °C. Details as described under Methods.

rhodamine 6G dye laser as a pulsed excitation source, with single photon counting electronics and the decay analysis software provided at the Regional Laser Biological Laboratory at the Chemistry Department of the University of Pennsylvania.

^{31}P NMR. For ^{31}P NMR, several vesicle preparations were made and pooled (24 μmol of lipid) and subjected to ultracentrifugation at 200000*g* for 60 min. This resulted in sedimentation of all but a few of the very smallest vesicles. The pellet was resuspended in 1.2 mL of buffer. NMR spectra were obtained on an 8.45-T Bruker AM spectrometer operating at 145.8 MHz for phosphorus. Lipid samples were placed, under nitrogen, in a 5-mm sample tube, which was inserted into a coaxial 10-mm tube containing methanol for precise temperature control (Martin & Martin, 1980). Spectra were obtained in a 10-mm probe with a spin-echo sequence (90–τ–180–τ–AQ) and cyclops phase cycling using a refocusing delay of 25 μs. Typical acquisition parameters were as follows: 90° pulse, 7–10 μs; spectral window, 83 kHz; 1600 data points zero-filled to 4K; repetition time 2.5 s (largely relaxed spectra); 6.5-W scalar composite pulse decoupling during acquisition; 500 transients.

RESULTS

CD Spectroscopy. Previous studies have characterized the channel and nonchannel forms of gramicidin using CD spectroscopy [e.g., see LoGrasso et al. (1988), Killian and Urry (1988), and Killian et al. (1988a,b)]. The results have demonstrated that the solvent used for the gramicidin dictates whether the channel or nonchannel form will be obtained on codispersing with lipids even though the solvent is removed prior to codispersing with the lipids. In particular, the channel form has a characteristic positive ellipticity at ~229 nm. Therefore, CD spectroscopy was used to confirm that the channel and nonchannel forms were obtained using the phospholipid vesicle systems of the present study, according to the solvent history and heat treatment of the gramicidin.

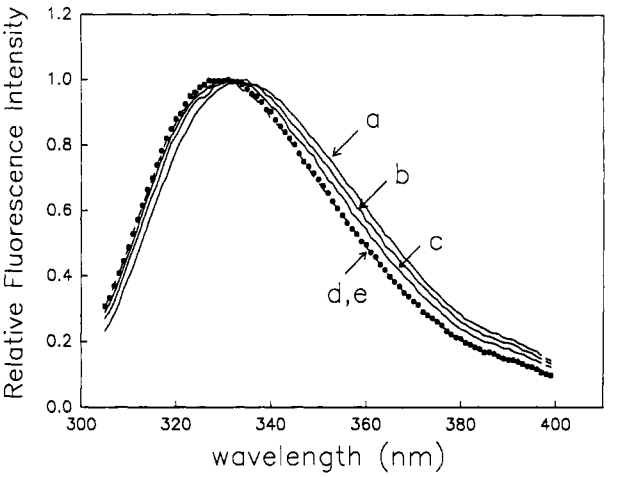

FIGURE 3: Effect of heating at 60 °C for different lengths of time on the fluorescence emission spectra of gramicidin/POPC (1:10 molar ratio) vesicles (50 μM) using 280-nm excitation. (a–c) Using chloroform/methanol (97:3 by volume, removed prior to codispersing); (b) vesicles heated for 6 h at 60 °C; (c) vesicles heated for 12 h at 60 °C; (d, e) using trifluoroethanol (removed prior to codispersing); (d) vesicles heated at 60 °C for 12 h. All samples were kept for 12 h before spectra were taken; samples which were heated at 60 °C for less than 12 h were stored at 4 °C for the balance of the 12 h. To obtain the emission maxima, emission spectra were obtained after the stated time, the spectra were fitted to a polynomial nonlinear least-squares curve, and the emission maxima were determined. All measurements were at 37 °C. Other details are as described under Methods.

The spectra shown in Figure 2 for the solvent chloroform/methanol or for ethanol closely resembled the spectra for the nonchannel form of gramicidin, as previously shown (LoGrasso et al., 1988; Killian & Urry, 1988; Killian et al., 1988a,b). After heating at 60 °C for 12 h, or alternatively if the solvent trifluoroethanol was used, spectra characteristic of the channel form of gramicidin were obtained. Heating of the vesicles containing gramicidin (made using trifluoroethanol as the solvent) did not appreciably affect the spectra. From these results, it was concluded that the preparations used in this work were of the nonchannel form if chloroform/methanol or ethanol was used as the solvent and were of the channel form when trifluoroethanol was used. The preparations are referred to as channel and nonchannel forms, respectively, for the remainder of this paper.

Tryptophan Fluorescence. Having confirmed that the gramicidin conformations were as expected according to the solvent history and heat treatment received, the fluorescence emission properties of the gramicidin tryptophans were then examined. The fluorescence emission spectra of the nonchannel and channel gramicidin tryptophans in POPC vesicles are shown in Figure 3. A clear difference in the emission maxima is shown, with the nonchannel form being several nanometers to the red of the channel form. After the vesicles were heated, the emission maxima of the nonchannel form of gramicidin gradually shifted toward the blue. Spectra taken after 0, 6, and 12 h of heating are shown in Figure 3a–c. It can be seen that after 12 h of heating the emission spectra of

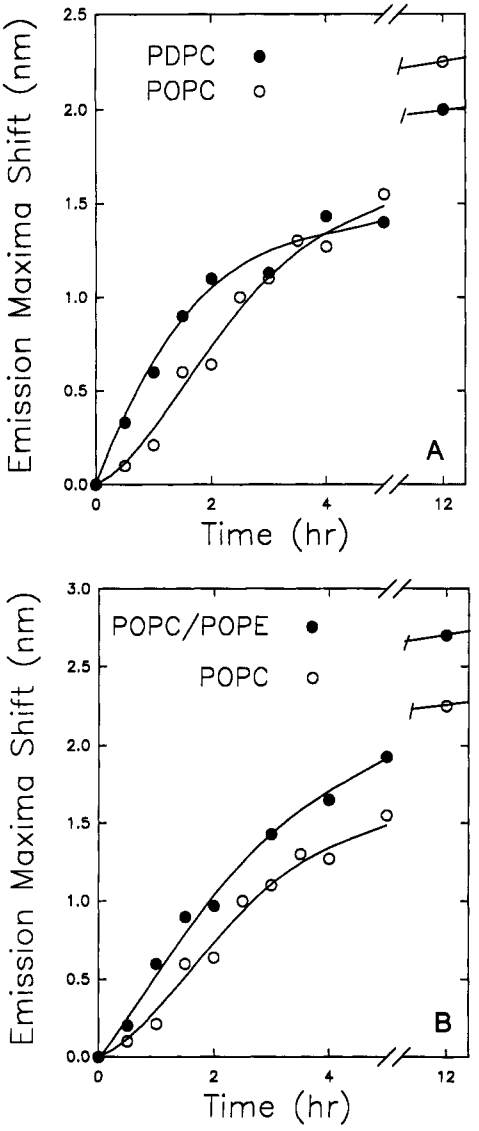

FIGURE 4: Effect of heating at 60 °C for different lengths of time on the fluorescence emission maxima (measured at 37 °C), obtained upon excitation at 280 nm, for gramicidin/phospholipid (1:10 molar ratio) vesicles made using chloroform/methanol (97:3 by volume, removed prior to codispersing). (A) comparison of POPC (open circles) with PDPC (closed circles); (B) comparison of POPC (open circles) with POPC/POPE (closed circles) (4:1 molar ratio). Other conditions were as in Figure 3.

the nonchannel and those of the channel form of gramicidin were nearly identical. Heating of the vesicles with the channel form of gramicidin had no effect on the fluorescence emission spectra (compare scans d and e of Figure 3); also, heating of the channel form had no effect on the CD spectra (Figure 2C). Lower temperatures for the nonchannel to channel conversion were investigated, but it was found that for the conversion to occur in a reasonable time 60 °C was optimal. Note that the CD spectra were taken at 25 °C whereas the fluorescence spectra shown in Figure 3 were taken at 37 °C. The differences in temperature had no effect on the conclusions although fluorescence spectral measurements were also taken at 25 °C to verify this (not shown).

The extent of the emission maxima shift with time, indicating the conversion of the gramicidin from the nonchannel to channel forms, was compared for gramicidin reconstituted in POPC, POPC/POPE, and PDPC. PDPC has a polyunsaturated *sn*-2 phosphatidylcholine chain containing six cis-double bonds. The extent of the emission maxima shift due to heating was found to be greater for gramicidin/PDPC

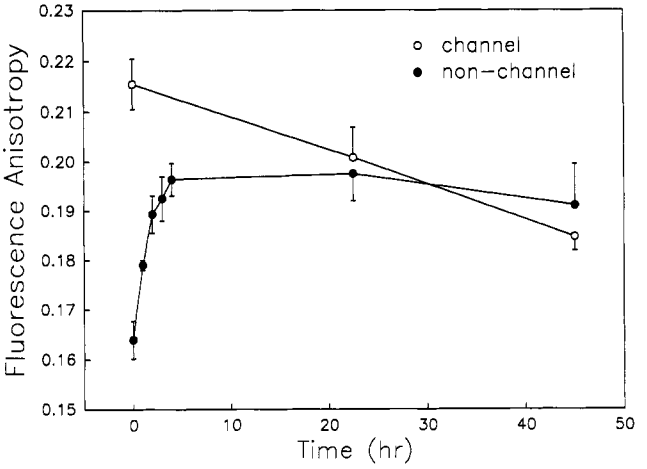

FIGURE 5: Effect of heating at 60 °C for different lengths of time on the steady-state fluorescence anisotropy obtained upon excitation at 280 nm with the emission observed at 340 nm using a 10-nm band-bass interference filter (measured at 37 °C) after heating at 60 °C. Vesicles (50 μM) were made from gramicidin/POPC (1:10 molar ratio) using chloroform/methanol (97:3 by volume, removed prior to codispersing). Details as described under Methods.

compared to gramicidin/POPC during the earlier 0–4-h heating period as shown in Figure 4A.

To determine the effect of PE on the rate of the nonchannel to channel conversion, as shown by the fluorescence emission maxima blue shift, a mixture of POPC/POPE (4:1, molar) was compared to POPC alone. The results shown in Figure 4B reveal that PE causes an accelerated emission shift, especially at the earlier times of heating, with the extent of the shift with PE being twice that of PC alone at 1 h. Since the fatty acyl composition is the same for POPC and POPE, it may be assumed that the PE head group was responsible for the effect.

The fluorescence anisotropy of the gramicidin tryptophan in gramicidin/POPC vesicles was also determined. The results in Figure 5 show that the channel form of gramicidin has a considerably higher anisotropy compared to the nonchannel form. After the nonchannel form of gramicidin was heated at 60 °C, there was a time-dependent increase in the fluorescence anisotropy as it converted to the channel form. By contrast, the channel form showed a slight decrease after the heating. After 12 h, the fluorescence anisotropy values of both conformations were practically identical.

A time-resolved fluorescence decay analysis of the gramicidin tryptophans in a lyso-PC micelle preparation has been previously reported (Masotti et al., 1986). The decay showed a complexity typical of tryptophan-containing proteins and revealed at least three components. To determine the fluorescence decay properties underlying the differences in the channel and nonchannel fluorescence emission spectra in the present study, channel and nonchannel gramicidin conformations in POPC bilayers were compared. It was found that adequate analysis required a four-exponential fit to the data. For the POPC nonchannel gramicidin preparation (measured at 25 °C), the fluorescence lifetimes (nanoseconds; fractional amplitudes in parentheses) were as follows: 3.64 (0.38), 1.12 (0.46), 0.20 (0.13), and 0.004 (0.03). For the channel form, the lifetimes were 2.66 (0.24), 0.94 (0.57), 0.21 (0.15), and 0.01 (0.04). Thus, the larger lifetime value was considerably decreased in the channel form as compared to the nonchannel form with the other parameters being similar. The complexity of the decay is not surprising since gramicidin contains four tryptophans and these will interact with each other on the nanosecond time scale.

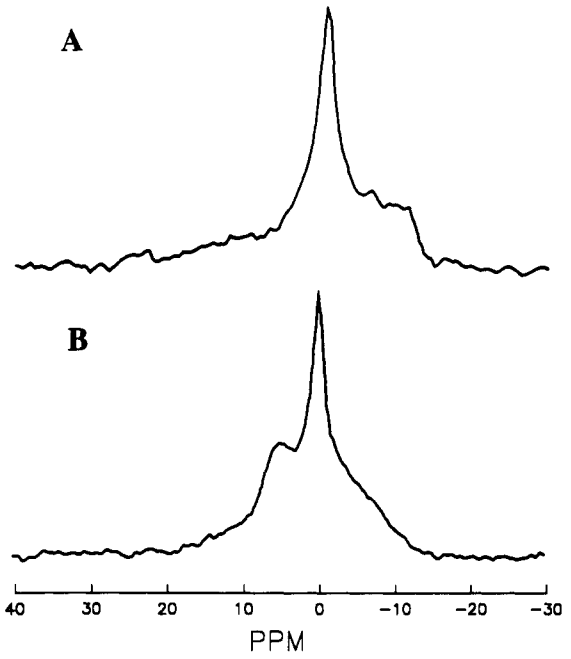

FIGURE 6: ^{31}P NMR spectra of gramicidin/POPC (1:10 molar ratio) codispersed from (A) chloroform/methanol (97:3 by volume) and (B) trifluoroethanol (solvents removed prior to codispersion), taken at 37 °C. Details as described under Methods.

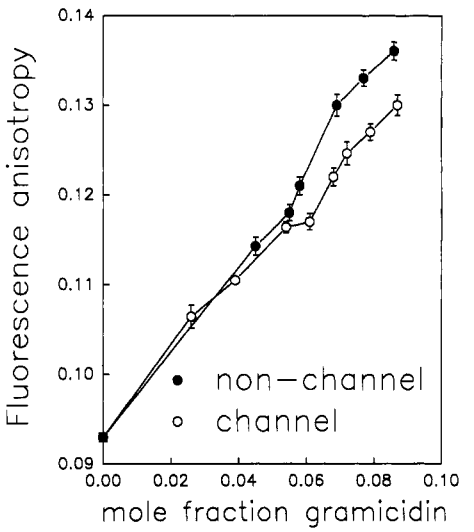

FIGURE 7: Steady-state fluorescence anisotropy of DPH (excitation 360 nm, emission 10-nm band-pass interference filter at 430 nm) in gramicidin/POPC (50 μM) codispersed after chloroform/methanol (97:3 by volume) (closed circles) and trifluoroethanol (open circles) (solvents removed prior to codispersion) to form the nonchannel and channel gramicidin conformations, respectively (gramicidin:phospholipid ratio 1:10). The vesicles were labeled with DPH by codispersing (DPH:lipid ratio 1:400). The determination of the gramicidin:phospholipid ratio and other details were performed as described under Methods.

^{31}P NMR. Also, although larger vesicles of the multilamellar type would have been more ideal for the ^{31}P NMR measurements, the intention was to examine vesicles prepared by the same method as for the fluorescence measurements. However, the method leads to a vesicle suspension which is too dilute for ^{31}P NMR. The vesicles were therefore subjected to centrifugation as described under Methods. A potential problem with this approach is that the sonication step tends to produce small vesicles and a fraction of these vesicles may have been lost if they failed to pellet as a result of centrifugation. However, the loss would have been negligible and in any case would have resulted in only a slightly diminished 0 ppm signal, with no effect on the signal in the region of the spectrum where the H_{II} phase would be expected to contribute (the region in question); thus, it could not affect the conclusions drawn. Also it is likely that because of the high protein:lipid ratio, the degree of curvature normally found in small unilamellar vesicles may have been unattainable, thus further minimizing this loss.

The spectra in Figure 6 shown for channel and nonchannel gramicidin in POPC vesicles reveal the presence of small vesicles, indicated by a resonance near 0 ppm (isotropic). This would be expected since the preparation used a sonication step. The isotropic ^{31}P NMR spectral resonance could be due to micellar structures; however, the broadness of the spectra makes this highly unlikely and indicates a bilayer configuration for vesicles with a range of sizes. An underlying signal arising from larger multilamellar vesicles (high-field shoulder) is also discernible. In the vesicles containing the channel form of gramicidin, a small amount of H_{II} lipid is apparent, as indicated by the slight low-field shoulder. Since the nonchannel to channel conversion was performed at 60 °C, ^{31}P NMR spectra were also obtained at this temperature. The spectra did not show any appreciable differences from those taken at 37 °C (data not shown). Thus, it appears the vesicle preparations were heterogeneous with respect to size and consisted largely of bilayer lipids.

DPH Fluorescence Anisotropy. The lipid order for vesicles containing channel and nonchannel gramicidin was determined using the fluorophore DPH. The fluorophore was incorporated by codispersing with the gramicidin and lipid. The fluorescence anisotropy for different protein:lipid ratios is shown in Figure 7. For higher protein:lipid ratios, it can be seen that the fluorescence anisotropy of DPH was higher for vesicles containing the nonchannel as compared to the channel forms of gramicidin. This indicated that the lipid order was higher for the lipid bilayer containing the nonchannel gramicidin and that it was affected differently according to the gramicidin conformation. For progressively lower protein:lipid ratios, the fluorescence anisotropy for the channel and nonchannel forms converged. This would be expected since for the lower protein:lipid ratio an increasing proportion of the DPH was reporting from the bulk lipid region which would be expected to be the same for two preparations.

Discussion

In this study, the properties of gramicidin in bilayers of unsaturated lipids were investigated. The previously established dependence of the gramicidin solvent type on its conformation obtained on subsequent codispersing with lipids was utilized in the present study. Characterization of the gramicidin/lipid bilayers by CD confirmed that both channel and nonchannel forms of gramicidin can be reconstituted into POPC bilayers, as previously demonstrated for disaturated and diunsaturated phospholipids (LoGrasso et al., 1988; Killian & Urry, 1988; Killian et al., 1988a,b). When lipid bilayers containing the nonchannel form are subjected to heating, the nonchannel double-helical dimer first uncoils to yield helical monomers, which then reassociate to form single-helical dimers spanning the bilayer and forming the ion channel. In this work, we have shown that the interconversion can be followed by monitoring the fluorescence of gramicidin and that the adjacent lipid chain motion is also sensitive to gramicidin conformation.

While the fluorescence emission properties of gramicidin have been determined in previous studies, these have been performed with lyso-PC micelle systems, rather than in the monounsaturated phospholipid bilayer systems of the present

work. In addition, at the time the previous studies were carried out, the solvent history dependence of gramicidin reconstitution had not been properly delineated as it has been recently (LoGrasso et al., 1988; Killian & Urry, 1988; Killian et al., 1988a,b; Bano et al., 1989, 1991). In the study of the incorporation of gramicidin into lyso-PC micelles (Masotti et al., 1986), CD spectra indicated that after only 90 min of heating of the nonchannel form, the channel form predominated with no further change occurring. The fluorescence emission maxima of the gramicidin tryptophans also changed, with a ~5-nm shift toward shorter wavelengths; the shift was, however, only just visible after 4 h and was not complete until as much as 24 h of incubation. CD spectra showed a form which, in light of more recent studies, is clearly recognizable as the nonchannel form, converting after heating at 70 °C for 90 min into a channel structure. The fluorescence emission spectra also shifted to shorter wavelengths, but this took 24 h with very little shift at 60 min. Thus, from these studies it was concluded there was no apparent correlation between the fluorescence changes and the nonchannel conformational change. These results contrast with the present study (using CD and fluorescence measurements) for monounsaturated lipids, and for disaturated and diunsaturated lipids using CD spectroscopy elsewhere (LoGrasso et al., 1988; Killian & Urry, 1988; Killian et al., 1988a,b). First, as shown by CD in the present study, the nonchannel to channel conversion for the bilayer systems, produced by heating at 60 °C, was not complete until 10–12 h, a longer time scale than for the lyso-PC micelle study (Masotti et al., 1986). Also, since the gramicidin was introduced as a powder in the later report, the slow incorporation into the lipid micelles was an additional factor to consider. Very recently, a slow nonchannel to channel conversion rate has also been confirmed using an HPLC technique (Bano et al., 1991). This raises the question of whether the conversion of the nonchannel to the channel form and the tryptophan spectral changes are two aspects of the same process or if they are independent features. This is an important question, bearing in mind the essential requirement of the tryptophans for channel functioning. The results of the present study and the HPLC study both show similar slow time-dependent changes, so that it would appear that the two are different aspects of the same process.

In this study, the CD and fluorescence spectral changes resulting from heating of the nonchannel form of gramicidin in lipid bilayers occurred in parallel. There were some small differences in that the fluorescence emission maxima of the nonchannel form heated for 12 h were not quite equivalent to the unheated channel preparation, although after 48 h the spectra were almost indistinguishable (results not shown). In addition, although the fluorescence emission spectra of the channel form were not affected by heating, the fluorescence anisotropy did show a slight decrease; however, after 12 h, the fluorescence anisotropies of the channel and heat-treated nonchannel forms were very close. Thus, the results support the contention that the tryptophan fluorescence emission changes can be used to monitor the heat conversion of the nonchannel to channel gramicidin conformations. This is further supported by a recent suggestion (LoGrasso et al., 1988) that the tryptophans may have an important role in the CD spectral changes. It was inferred that the only chromophores present in gramicidin which could account for the spectral differences for gramicidin in trifluoroethanol and chloroform/methanol are the indole rings and the amide groups of the peptide backbone. This would either be due to a major difference in the protein backbone or be due to a change in the relative orientation of the indole rings. NMR data showing that at least six sites along the polypeptide backbone possess the same conformation further support this contention. Also, various aspects of the CD spectra have been attributed to the tryptophan residues (Masotti et al., 1980; Urry et al., 1985).

The gradual nature of tryptophan spectral shifts might imply intermediate structural forms for the gramicidin. However, it has recently been shown that using solvents known to favor the nonchannel conformation, conductance properties of the channel form were still detectable (Sawyer et al., 1990) even though the physical studies appear to show only nonchannel structures. It was suggested that the idea that channel and nonchannel forms may be obtained in lipid bilayers strictly according to the solvent history may therefore be invalid. A more likely interpretation is that, even without heating, a nonchannel preparation contains at least some channel conformations. While the channel structures in the nonchannel preparation may be too few to detect by physical methods, for the conductance properties only a very small proportion of channel structures would be needed. This consideration leads us to propose that instead of a gradual transition from a nonchannel to channel conformation, the gramicidin will spontaneously convert from one conformation to the other during heating. Thus, the longer the period of heating, and the higher the temperature, the more likely the gramicidin conformational conversion becomes. Therefore, the spectra of intermediate states would arise from a mixture of the two conformations.

The tryptophan spectral maxima shifts, as the nonchannel form converted to the channel form for gramicidin in PDPC vesicles, were initially more rapid compared to POPC. Since the lipid order in PDPC bilayers is considerably less than for POPC (Nie et al., 1989), then presumably this allows a greater freedom for the uncoiling of the gramicidin helical dimer nonchannel form to occur within the bilayer, followed by the recoiling of the monomers into individual helices and finally their head-to-tail dimerization forming the channel conformation. A similar result has been shown using CD, for shorter chain disaturated lipids, e.g., dilauryl-PC (Killian et al., 1988b).

Although there have been many studies of the conformation of gramicidin in various lipid systems, there have been very few on the effect of gramicidin on lipid order. Of the studies which have addressed this issue [see Rice and Oldfield (1979), Chapman et al. (1977), and Macdonald and Seelig (1988)], none have shown the effect of the channel and nonchannel conformations on lipid order. Recently, the rotational properties of the gramicidin tryptophans were studied in a number of systems, including DOPC and egg PC bilayers, where the solvent used would suggest that a nonchannel conformation was obtained (Scarlata, 1988). It was found that tryptophan–tryptophan ring-stacking interactions were limiting the ability of the tryptophan rotations to respond to temperature and viscosity changes and that in turn the latter effects could be modulating the inter- and intramolecular gramicidin interactions. In the present study, lipid order in both channel and nonchannel forms of gramicidin was investigated using the fluorescence anisotropy of the lipid probe DPH, which relates mostly to order and to some extent to the rate of motion. The results showed a higher order for the nonchannel, as compared to the channel, gramicidin conformation, for the same protein:lipid ratios. At the present time, the reason for this is not known, mainly because the disposition of the nonchannel in the lipid bilayer has yet to be determined. The

results do, however, provide another approach for distinguishing between the two forms and their interconversion.

In studies where gramicidin was added to preformed bilayers by injection from different solvents, a solvent-dependent formation of H_{II} lipids was shown (Tournois et al., 1987). Addition of gramicidin from ethanol led to a bilayer form of DOPC, whereas trifluoroethanol and DMSO led to H_{II} lipids, suggesting that the channel form is H_{II}-promoting. In the present study, where the solvents were removed prior to codispersing with the lipids, ^{31}P NMR spectra revealed a minor H_{II} peak for the channel form (for POPC and using trifluoroethanol as the solvent) which would support the idea that the channel form tends to promote H_{II}. Thus, one might expect that H_{II}-promoting lipids would induce the nonchannel to channel conversion, possibly even if the system is below the bilayer to hexagonal phase transition temperature. One class of lipids which is known to do this is phosphatidylethanolamine. The results of the present study would support this idea since it was found that POPE increased the rate of the nonchannel to channel conformational transition, even at the temperature (60 °C) the lipids were in the bilayer phase.

It has previously been pointed out that energetically unfavorable conformations of gramicidin may in effect be "stored" in a lipid bilayer and that this may serve as a useful model for membrane proteins in general (LoGrasso et al., 1988). In this study, it has been shown that tryptophan spectral properties can be used to follow such conformational transitions, which are also reflected in adjacent lipid chain order.

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