Gramicidin A/Short-Chain Phospholipid Dispersions: Chain Length Dependence of Gramicidin Conformation and Lipid Organization†

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ABSTRACT: Gramicidin–lipid interactions were investigated using diacylphosphatidylcholines that contained two identical acyl chains of varying length, between 6 and 14 carbons. The gramicidin A (gA) conformation was monitored by circular dichroism (CD) spectroscopy and high-performance size-exclusion chromatography, and the lipid organization was investigated using ^{31}P and ^{1}H NMR spectroscopy and negative-stain electron microscopy. Diacylphosphatidylcholine (PC) lipids with chain lengths between 4 and 8 carbons have been previously shown to have a micellar organization in aqueous solution [Lin, T.-L., et al. (1986) *J. Am. Chem. Soc. 108*, 3499–3507]. CD spectra of aqueous gA/lipid dispersions, at a ratio of 1:28, demonstrated that the channel conformation of gA can be readily obtained when the acyl chain length is ≥10, but not when the chain length is ≤7. Size-exclusion chromatography revealed that the fraction of gA that could easily be dissociated into monomers in the dispersions increased with increasing acyl chain length, in agreement with the CD results. For a chain length of 8, the results were intermediate. The formation of the channel structure was found to depend on the "solvent-history", the temperature, the gA and lipid concentrations, the gA:lipid ratio, and consequently on the method of sample preparation. ^{1}H and ^{31}P NMR results suggest that codispersed gA increases the size of dioctanoyl-PC aggregates, but not of dihexanoyl-PC micelles. Negative-stain electron microscopy directly supports these findings. Dihexanoyl-PC (28 mM) was able to solubilize 1 mM gA in H_2O, but the gA was not in the "channel" conformation. By contrast, dioctanoyl-PC profoundly influenced the conformation of gA, inducing the "channel" conformation that is typically observed in (longer-chain) bilayer lipids. At the same time, gA influenced the dioctanoyl-PC, increasing the size of the lipid aggregates, as well as inhibiting its tendency toward phase separation. The gramicidin–lipid interactions are therefore reciprocal.

Gramicidin A (gA),[1] a hydrophobic, channel-forming peptide with an alternating L,D amino acid sequence, has been shown to act as a potent modulator of the structure of both monoacyl- and diacylphospholipids (Killian & De Kruijff, 1986; Killian et al., 1987; Killian, 1992). Due to its ability to form channels in model membranes, it has been used extensively as a prototype transmembrane channel (Andersen & Koeppe, 1992) and as a model for a hydrophobic segment of membrane proteins (Chapman et al., 1977; Rice & Oldfield, 1979). gA is produced by *Bacillus brevis* during sporulation (Hotchkiss & Dubos, 1940) as the major component of a mixture of linear pentadecapeptides (Sarges & Witkop, 1965). The gA sequence is formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. This paper concerns the interaction of gA with short-chain phospholipids.

Upon incorporation into certain lipids, gA can induce changes in the organization of the lipids. For example, the addition of gA to MPPC, which has a single acyl chain and by itself prefers a micellar organization, induces the formation of an extended lamellar lipid structure (Spisni et al., 1983; Killian et al., 1983). In bilayer-forming diacylphosphatidylcholines, when the chain length is longer than 16 carbons, gramicidin induces a transition from a bilayer to a hexagonal H_{II} configuration (Van Echteld et al., 1982). The lipid-modulating activity of gramicidin is dependent upon its tertiary structure, in particular the $\beta^{6.3}$-helical "channel" conformation (Killian et al., 1988; Tournois et al., 1987). The changes in lipid organization can be monitored by negative-stain electron microscopy and by ^{31}P NMR spectroscopy (Cullis & De Kruijff, 1979).

The conformational behavior of gA is complex and is dependent upon its environment (Killian et al., 1988; Killian, 1992). In nonpolar solvents such as THF, the predominant conformer at equilibrium is a double-helical dimer, whereas in more polar solvents such as TFE a monomeric form predominates (Bañó et al., 1988). In biological or model membrane systems, gA adopts a transmembrane channel conformation which allows the passage of water and small, monovalent cations. This conformation is a right-handed, formyl-NH to formyl-NH dimer of $\beta^{6.3}$-helical monomers in which the hydrophobic side chains are located on the external face of the channel, in contact with the lipid acyl chains (Urry, 1971). In "dilute" systems (very low concentrations and gA:lipid ratios), gA channels are readily observed [e.g., see Sawyer et al. (1989)]. For more concentrated systems and high gA:lipid ratios (1:100–1:5), the degree of $\beta^{6.3}$-channel formation depends on numerous experimental parameters including, but not limited to, the type of organic solvent used to dissolve the peptide, the lipid to peptide ratio, the time of sonication, and the temperature (Killian et al., 1988; Bañó et al., 1992). A unique CD spectrum for gA as a $\beta^{6.3}$-helical channel enables CD to be used to monitor changes in conformation. A given

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[1] Abbreviations: gA, gramicidin A; PC, phosphatidylcholine; MPPC, monopalmitoylphosphatidylcholine; CD, circular dichroism; cmc, critical micelle concentration; di-C_n-PC, diacyl(C_n)phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; TFE, trifluoroethanol; THF, tetrahydrofuran; SE-HPLC, size-exclusion high-performance liquid chromatography.

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CD spectrum is the result of the individual contributions of both $\beta^{6.3}$- and double-helical gA molecules, and an increasingly negative ellipticity near 230 nm indicates a higher fraction of double-helical dimers (Bañó et al., 1991). The fraction of gA present as the "non-channel" (double-helical)[2] form can also be determined by size-exclusion HPLC. Under conditions where the lipids are disrupted, the $\beta^{6.3}$ channel, which is stabilized by only six intermolecular H bonds, will dissociate and elute as monomers (Bañó et al., 1988). In contrast, the double-helical dimers, which are stabilized by 28 intermolecular H bonds, will remain intact and elute as dimers.

It has previously been shown that unlike long-chain diacylphosphatidylcholines which form vesicles in aqueous solution, the corresponding short-chain diacyl lipids with chain lengths between 4 and 8 carbons form micelles (Tausk et al., 1974). The properties of the short-chain lecithins, such as cmc, average size, and shape, are dependent on the acyl chain length (Lin et al., 1987). At a given concentration, an equilibrium exists between ellipsoidal micelles of varying lengths, with the average length being proportional to the aggregation number. Dihexanoyl-PC (di-C_6-PC) forms a prolate ellipsoid micelle with an aggregation number of 19 ± 1 when the concentration of the lipid is between 27 and 360 mM (Lin et al., 1986). The cmc for dihexanoyl-PC is 12–14 mM (Roberts et al., 1978). Diheptanoyl-PC (di-C_7-PC) forms rod-shaped micelles, with a minimum aggregation number of 27 and a cmc of 2 mM (Hershberg et al., 1976); these micelles increase in length with increasing lipid concentration (Tausk et al., 1974). The largest lecithin in the series, dioctanoyl-PC (di-C_8-PC), has a cmc of 0.3 mM and forms very large, concentration-dependent micelles above this concentration (Burns et al., 1983).

This paper reports investigations of the effects of short-chain lipids on the stability and conformation of gA in aqueous solution, and of gA conformation on the micellar organization of the short-chain lipids. To address these questions, gA/lipid dispersions were prepared using di-C_n-PC's with n between 6 and 12 carbons. The results indicate that the channel conformation can be achieved only if $n \geq 8$ and that the incorporation of gA into a lipid micelle as the $\beta^{6.3}$ channel correlates directly with a change in lipid organization.

EXPERIMENTAL PROCEDURES

Materials. Linear gramicidin (gramicidin D) was purchased from Sigma Chemical Co. (St. Louis, MO). Diacylphosphocholine lipids dihexanoyl (C6), diheptanoyl (C7), dioctanoyl (C8), didecanoyl (C10), dilauroyl (C12), and dimyristoyl (C14) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Methanol and chloroform (HPLC grade) were from Burdick and Jackson (Muskegon, MI). Deuterium oxide (D_2O), 99.9%, and praseodymium(III) nitrate hexahydrate, 99.99+%, were from Aldrich Chemical Co. (Milwaukee, WI). Water was deionized Millipore Corp. Milli-Q water (Bedford, MA).

Gramicidin A was purified from gramicidin D by preparative reversed-phase HPLC (phenylsilica column packing) as previously described by Koeppe and Weiss (1981).

Sample Preparation. gA/lipid dispersions were prepared by a procedure adapted from Barrow and Lentz (1980); 140 nmol–50 μmol of lipid was added from stock methanol solutions to dried gramicidin (10 nmol–1 μmol). The volume was brought to 200 μL by the addition of methanol/chloroform (50:50). The suspensions were mixed well by vortexing, and dried under vacuum overnight to remove all trace of solvent. The dried gramicidin/lipid mixtures were resuspended in 1 mL of N_2-flushed, filtered H_2O, sonicated for 1 min, incubated for 30 min, and then sonicated again for 30 min (all at 55 °C) using a Branson W-185 cell disrupter (power level 5) fitted with a Model 431-A cup horn accessory. Some samples were heated at 68 °C for 24 h as noted; these were then cooled to room temperature and treated as all the other samples. Samples were centrifuged at 12 500 rpm for 5 min at room temperature. The concentrations of gA in the supernatants were determined by measuring the absorbance at 280 nm using an extinction coefficient of 20 840 M^{-1} cm^{-1} (Turner et al., 1983). CD measurements were obtained at room temperature using a Jasco 710A spectrometer. Each spectrum is an average of six scans, using path lengths of 0.01 or 1 cm.

Size-Exclusion HPLC. Samples were prepared as above. The fraction of double-stranded versus single-stranded gA in each of the lipid suspensions was determined at room temperature according to the method of Bañó et al. (1988).

^1H and ^{31}P NMR. gA/lipid suspensions (1:28 ratio) were prepared as above, except that D_2O (10%) was added and the suspensions were filtered through 0.22-μm Millex-GV filters (Millipore) and transferred to 5-mm tubes. NMR spectra were recorded at 55 °C using a Varian VXR-500 spectrometer, with transmitter presaturation of the ^1H_2O resonance (for the ^1H spectra). ^{31}P NMR spectra were obtained under ^1H broadband decoupling conditions. For some samples, 10 mM $Pr(NO_3)_3$ was added after sonication. As a control, one of the samples was not sonicated.

Negative-Stain Electron Microscopy. Ten microliters of a sonicated lipid or lipid/gA suspension (prepared as above) was placed on a Formvar carbon-coated copper grid for 2 min at room temperature. The grid was washed with 10 μL of H_2O and stained with 2% aqueous uranyl acetate for 15 s. The grids were examined with a Joel 100CX electron microscope using 20000× magnification and tobacco mosaic virus as an external standard. For determination of the size distribution in each plate, at least 25 particles were measured on a 5 × 7 in. print.

RESULTS

Dependence of Gramicidin Conformation on Lipid Acyl Chain Length

CD Spectroscopy. The ability of short-chain phospholipids to induce the $\beta^{6.3}$-channel conformation of gA was investigated by CD spectroscopy. The channel conformation of gA is characterized by a unique CD spectrum with a maximum at 220 nm, a minimum at 230 nm, a positive peak at 235 nm, and negative ellipticity below 205 nm (Urry et al., 1979; Killian et al., 1988). It has previously been demonstrated that the channel conformation can be obtained when the acyl chain length is ≥12 carbons (Killian et al., 1988). To determine whether the channel conformation can be achieved in a short-chain phospholipid, gA/lipid dispersions at 0.5–1 mM gA concentration and a 1:28 gA:lipid ratio were prepared using the di-C_n-PC lipids. Figure 1 depicts the CD spectra of the dispersions for n = 6, 7, 8, 10, and 14.

A CD pattern characteristic of the $\beta^{6.3}$-helical structure was not observed when the acyl chain length was <8. Varying the gA concentration between 10 μM and 1.3 mM, while maintaining the 1:28 gA:lipid ratio, had no observable effect on the spectra for n = 6 or 7. In all cases when $n < 8$, the CD spectra were characterized by a large negative peak at

[2] Though a variety of double-helical conformations have been observed (Veatch & Blout, 1974), we will consider them as a group to be "non-channel"-form dimers.

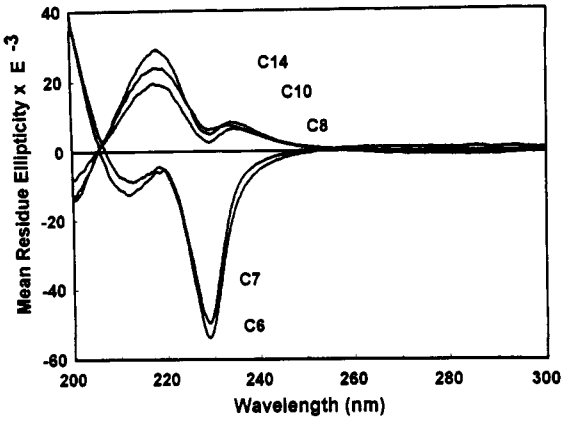

FIGURE 1: CD spectra of aqueous gA/di-C_n-PC dispersions for n = 6, 7, 8, 10, and 14. (Conditions: 1:28 gA:lipid ratio, 0.01-cm path length, 1 mM gA, 55 °C.)

229–230 nm, a smaller negative peak at 212 nm, and positive ellipticity below 208 nm. This type of spectrum is similar to that obtained when gA is predominantly present as a double-helical dimer (Wallace et al., 1981; Bañó et al., 1989).

The channel conformation could be achieved in the di-C_8-PC lipid dispersion; however, the conformation was particularly sensitive to the gA and lipid concentrations, as well as to the "solvent history" of the sample. In order to obtain the $\beta^{6.3}$-helical conformation in a di-C_8-PC lipid suspension, it was essential that all traces of organic solvent be removed from the gA/lipid film before water was added. To ensure that the film was completely dry, the samples were routinely dried under vacuum (0.05 mmHg, maintained by continuous pumping in the presence of a liquid nitrogen trap) for 24–36 h. If the samples were insufficiently dried, an intermediate spectrum was observed. This suggested a mixture of conformers, most likely consisting of double helices along with $\beta^{6.3}$ helices.

The $\beta^{6.3}$-helical conformation was more readily obtained when gA was dispersed with di-C_{10}-PC. In a di-C_{10}-PC lipid dispersion, the gA conformation was less sensitive to traces of solvent, and therefore could be obtained after drying the gA/lipid film for as little as 4 h. For both the di-C_8- and the di-C_{10}-PC lipids, when the gA concentration was decreased to 10 μM while maintaining the gA:lipid ratio at 1:28, a mixture of conformers was again observed. At the lower concentration, the ellipticity at 230 nm was more negative with di-C_8-PC than with di-C_{10}-PC. Since 280 μM is close to the cmc of these lipids ($\sim$300 μM for di-C_8-PC), where lipid micelles exist in equilibrium with lipid monomers, the presence of a mixture of gA conformations is not surprising. At 50 μM gA and a 1:28 ratio, the channel conformation was obtained for both di-C_8-PC and di-C_{10}-PC. For chain lengths between 12 and 14, the channel structure was readily obtained for both the low (10 μM) and high (1 mM) gA concentrations.

As it has previously been demonstrated that incubating gA/lipid dispersions at elevated temperatures for several hours often results in a change in conformation toward that of the $\beta^{6.3}$ channel (Urry et al., 1975; Shungu et al., 1986; Killian et al., 1988; Sawyer et al., 1990; Bañó et al., 1991), the gA/lipid dispersions were heated at 68 °C for up to 12 h. The CD spectra of the gA/di-C_7-PC and gA/di-C_{10}-PC dispersions were unchanged after heat treatment; the spectrum of the former remained negative ("non-channel" conformation), and that of the latter remained positive ("channel" conformation). The CD spectrum of gA/di-C_8-PC, however, was markedly changed *away from* the "channel" conformation after heating at 68 °C for 24 h (Figure 2). Prior to heating, the CD pattern

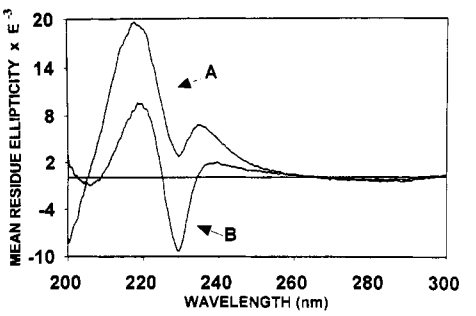

FIGURE 2: CD spectra of gA/di-C_8-PC heated for 12 h at (A) 55 °C and (B) 68 °C (conditions as in Figure 1).

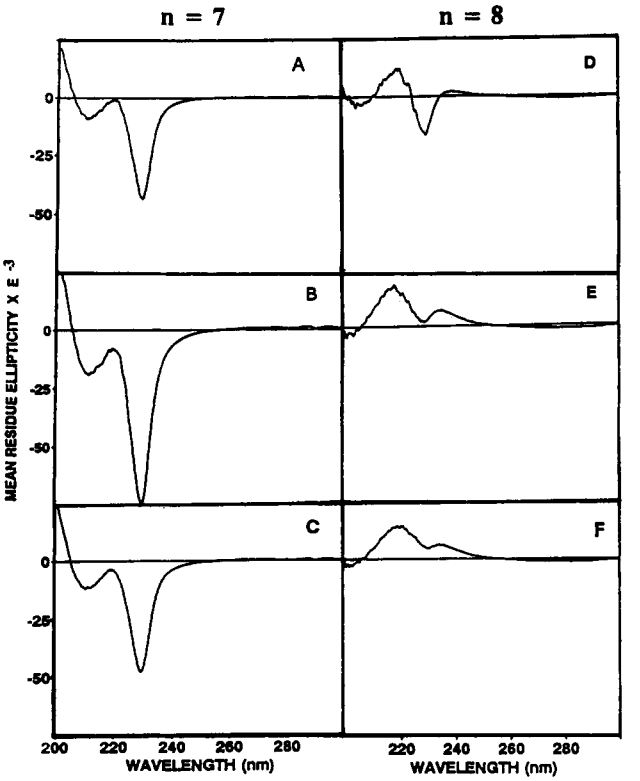

FIGURE 3: CD spectra of gA/di-C_7-PC at (A) 1:14, (B) 1:28, and (C) 1:50 gA:lipid ratios; and gA/di-C_8-PC at (D) 1:14, (E) 1:28, and (F) 1:50 gA:lipid ratios (conditions as in Figure 1).

suggested that gA existed predominantly in the $\beta^{6.3}$-channel conformation. In contrast, the heat-treated gA/di-C_8-PC, upon recooling to room temperature, exhibited an intermediate spectrum, suggesting the presence of a mixture of gA conformers (which then persisted at room temperature, and eventually precipitated).

Decreasing the gA:lipid ratio has also been shown to cause a conformational change in gA toward that of the $\beta^{6.3}$ helix (Killian et al., 1988). To test for this possibility, the gA:lipid ratio was varied between 1:14 and 1:50 for the di-C_7- and the di-C_8-PC dispersions (the gA concentration was $\geq$1 mM in all cases). The results are shown in Figure 3. The changes in ratio had no effect on the spectra of the gA/di-C_7-PC suspensions; the CD spectra were negative for all ratios tested (Figure 3A). For the gA/di-C_8-PC dispersions, an intermediate spectrum was obtained at a 1:14 ratio, whereas the channel spectrum was observed when the ratio was either 1:28 or 1:50 (Figure 3B).

All of the above observations—the sensitivity of the CD spectra of gA/di-C_8-PC dispersions to concentration, temperature, traces of solvent, and protein:lipid ratio—indicate that the n = 8 phospholipids provide a critical threshold environment for gA channel formation.

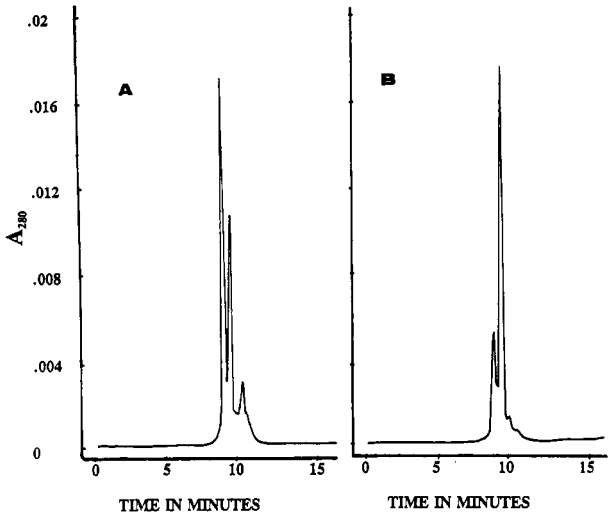

FIGURE 4: Size-exclusion HPLC elution profile of gA in (A) di-C_6-PC and (B) di-C_8-PC. Five microliters of a gA/lipid suspension was injected (1:28 gA:lipid ratio, 500 μM gA). Column, Ultrastyragel 1000 Å; mobile phase, THF; flow rate, 1.0 mL/min. [The third peak in (A) is due to the solvent. In (B), the solvent peak is not shown.]

High-Performance Size-Exclusion Chromatography. As a second test for the "channel" conformation, the gA/lipid dispersions were subjected to high-performance size-exclusion liquid chromatography. When several microliters of an aqueous gA/lipid suspension are injected onto a size-exclusion column which has been equilibrated with a nonpolar solvent such as THF, the phospholipid assemblies are disrupted by the organic solvent (Bañó et al., 1988); consequently, gA and lipids are released into the eluent. The $\beta^{6.3}$ channel, which is stabilized by only six intermolecular H bonds, dissociates and elutes primarily as monomers. In contrast, the double-helical dimer, which is stabilized by about 28 intermolecular H bonds, remains intact and elutes as a dimer (Bañó et al., 1988). The profiles in Figure 4 illustrate that about 62% of gA elutes as dimers when n = 6, but only about 22% when n = 8.

Previous studies using this method indicate that samples reflecting the characteristic "non-channel" CD pattern still may contain some monomer while those reflecting the characteristic "channel" spectra still may have some dimer present (Bañó et al., 1992). This pattern seems to be accentuated for the short-chain lipid samples. For the di-C_6/gA dispersion, which has a "non-channel" CD spectrum, double helices predominate, but still ≈38% of gA elutes as monomers. [The CD spectrum (Figure 1) suggests that these monomers are not $\beta^{6.3}$ helices; they could perhaps be unfolded monomers.] At 0.5 mM gA and a 1:28 gA:lipid ratio, the fraction of monomer increases with n to a maximum of 84% when n = 10. These results are consistent with the CD results (Figure 5) and have been observed in a time-dependent manner for other lipids (Bañó et al., 1992). The data clearly reflect an increase in the mass fraction of monomer when the acyl chain length is increased between 6 and 10 carbons.

Figure 5 illustrates how this trend correlates with an increase in the mean residue ellipticity at 229 nm in the corresponding CD spectra. As the lipid acyl chain length increases between 5 and 10 carbons, the CD pattern reflects a gradual transition toward that characteristic of the $\beta^{6.3}$-helical channel conformation. For n = 10–14, both the ellipticity at 229 nm and the percent monomer remain constant.

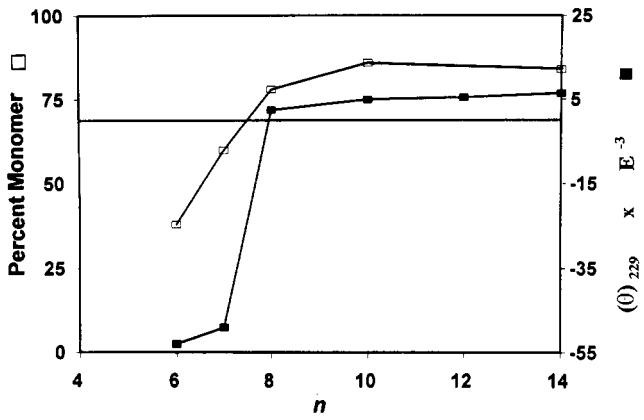

FIGURE 5: Comparison of the change in percent monomer from SE-HPLC, and in the ellipticity at 229 nm, versus acyl chain length (n) for gA/lipid dispersions. (Peptide and lipid concentrations as in Figure 1.)

Dependence of Short-Chain Phospholipid Organization on the Presence of $\beta^{6.3}$ "Channel-Form" Gramicidin

Loss of Phase Separation in gA/Di-C_8-PC. The di-C_8-PC dispersion remained opalescent even after sonication at 55 °C for 30 min, and phase separation occurred after several hours at room temperature or 55 °C. Upon addition of gA, the di-C_8-PC took on a cloudy, white appearance, and no phase separation was observed. Filtering the dispersion through a 0.2-μm filter had no effect on the turbidity or the gA concentration.

^1H NMR Spectroscopy. In order to determine whether the inclusion of gA in the lipid dispersions had an effect on the micellar organization of the lipids, the di-C_6-PC and di-C_8-PC dispersions were analyzed by ^1H NMR spectroscopy, both with and without gA present. The addition of gA to the di-C_6-PC lipid suspension did not result in any significant change in the ^1H NMR spectrum (Figure 6A,B). The relatively narrow resonances due to the lipid hydrogens remained uniformly narrow. [The resonance assignments in Figure 6A are from Roberts et al. (1978).] In contrast, the spectrum of the di-C_8-PC with gA added was quite different and much weaker than that of the lipid alone (Figure 6C,D). The intensities of all resonances were much reduced (note the peak heights relative to the residual H_2O peak in Figure 6C,D), and the resonances were broadened. The change in the ^1H NMR spectrum suggested that the incorporation of gA may have caused an increase in the size of the di-C_8-PC aggregates, resulting in a correspondingly slower rotational correlation time that broadened the ^1H resonances. No such effect was observed, however, for di-C_6-PC in the presence of gA. CD spectra confirmed that gA was present in the $\beta^{6.3}$-helical conformation in the di-C_8-PC sample, but not in the di-C_6-PC sample.

Electron Microscopy. The di-C_6- and the di-C_8-PC aggregates, alone and with gA present, were examined by negative-stain electron microscopy to provide visual information about the lipid structures. The electron micrographs are depicted in Figure 7. The micrograph of the di-C_6-PC lipid suspension alone demonstrates the presence of particles of 25–50-nm diameter (Figure 7A). Upon inclusion of gA in a di-C_6-PC suspension, the micrographs indicate that the particles are more uniformly sized and somewhat smaller (25–35 nm) than in the absence of gA (Figure 7B). In the presence of gA, the di-C_6-PC particles also appear aggregated with each other in the micrographs.

Particles of 25–50 nm were also observed in the micrograph of the di-C_8-PC lipid suspension without gA (Figure 7C).

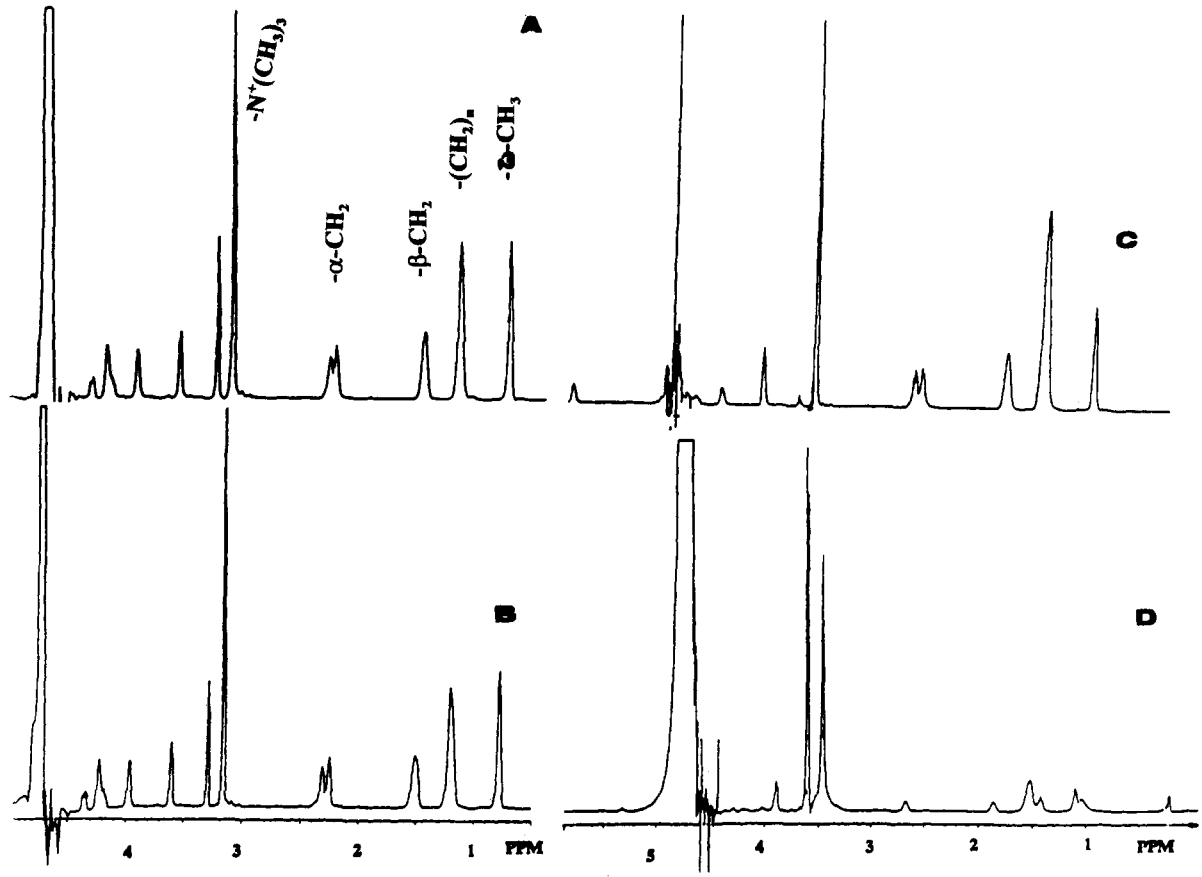

FIGURE 6: ^{1}H NMR spectra of (A) di-C_6-PC alone, (B) gA/di-C_6-PC, (C) di-C_8-PC alone, and (D) gA/di-C_8-PC (1 mM gA; 28 mM lipid, 55 °C). Selected spectral assignments are indicated in panel A; other assignments are given in Roberts et al. (1978).

Electron microscopy revealed, however, that when gA was included in this lipid suspension (and was present in the channel conformation, as confirmed by CD) the size of the di-C_8-PC aggregates increased significantly. Elongated particles with dimensions of about 100 × 150 nm (or more) are typical (Figure 7D). In addition, some of the lipid/gA particles seem to align end-to-end.

These micrographs directly support the ^{1}H NMR data, and confirm that the addition of gA to the di-C_8-PC dispersion causes a significant increase in the size of the lipid aggregates.

^{31}P NMR Spectroscopy. In order to investigate further the putative changes in lipid organization in di-C_8-PC, ^{31}P NMR spectra were recorded with and without gA present. The spectrum of the lipid alone showed a characteristic single sharp line (Figure 8A), which is due to rapid tumbling and lateral diffusion of the lipid molecules within the micelles that effectively averages the chemical shift anisotropy (Seelig, 1978). When gA was added to the di-C_8-PC dispersion, the resonance became much broader (not shown), suggesting a decrease in lipid motion. CD spectra confirmed that gA was present in the channel conformation. The line broadening upon incorporation of gA in the $\beta^{6.3}$-channel conformation suggested that lipid aggregation and/or bilayer formation might be occurring, as also suggested by the micrograph in Figure 7D.

Lanthenide shift reagents are able to distinguish between sealed vesicles and micelles (or linear bilayers). As a test for sealed vesicles, the ^{31}P NMR measurements were therefore repeated in the presence of 10 mM Pr^{3+}. It has been shown (Bystrov et al., 1972) that Pr^{3+} complexes with the phospholipid head groups of aqueous lipid dispersions, causing the resonances from exposed ^{31}P nuclei to be shifted downfield. The ^{31}P NMR spectra of di-C_8-PC alone, and after the addition of 10 mM Pr^{3+}, are shown in Figure 8A,B. The presence of the shift reagent results in a single, downfield-shifted resonance, confirming the presence of micelles. The spectrum of gA/di-C_8-PC in the presence of Pr^{3+} (Figure 8C) shows a single peak (now much broader) in the downfield position, indicating that again closed vesicles were not present. The significant line broadening suggested an increase in the size of the lipid aggregates, in agreement with the ^{1}H NMR and electron microscopy data (Figures 6D and 7D, respectively).

Because sonication has been found to aid in the incorporation of gramicidin (Shungu et al., 1986), as well as in the formation of vesicles from multilamellar bilayers (Huang, 1969), the gA/di-C_8-PC dispersion was sonicated for 30 min prior to addition of Pr^{3+}. The only change observed after sonication was a slight narrowing of the line width (Figure 8D). The gA/di-C_8-PC aggregates were therefore either large micelles or unsealed vesicles; they did not contain a population of ^{31}P nuclei that were inaccessible to Pr^{3+}.

DISCUSSION

gA exhibits complex interaction with its environment, whether solvent or lipid. The ability of gA to modulate the organization of lipids has previously been investigated using long-chain mono- and diacyl-PC's and has been shown to depend on the formation of the $\beta^{6.3}$ helix (Killian et al., 1988). We extend these observations to short-chain diacyl-PC's with identical acyl chains. The minimum acyl chain length to induce the $\beta^{6.3}$-channel conformation of gA in di-C_n-PC is n = 8. Concomitant with the incorporation of channel-form gA is an increase in the size of the di-C_8-PC lipid particles, from 25–50 to 100–150 nm.

When gA is added to MPPC micelles, dry or from an ethanol solution, a mixture of conformations is observed by CD (Masotti et al., 1980). Upon heating the gA/MPPC dis-

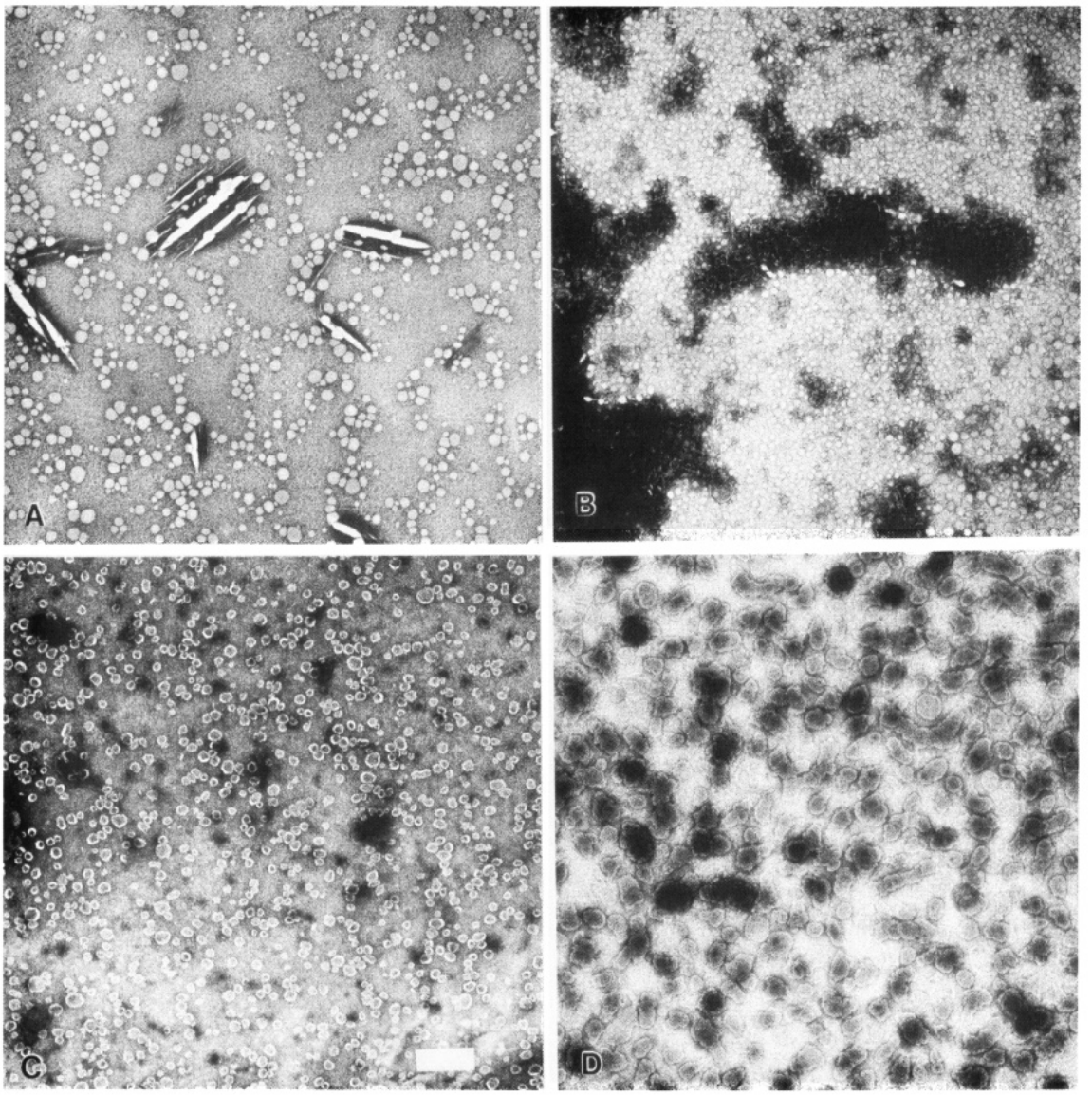

FIGURE 7: Electron micrographs of (A) di-C_6-PC, (B) gA/di-C_6-PC, (C) di-C_8-PC, and (D) gA/di-C_8-PC. Bar = 250 nm; 2% uranyl acetate. (Conditions as in Figure 6.)

persion, the CD spectrum reflects a change in the gA conformation to that of the $\beta^{6.3}$ helix, which is the channel conformation, and the lipid/gA complex reorganizes (partially) into bilayers (Urry et al., 1983; Killian et al., 1983, 1988; Spisni et al., 1983). Furthermore, the ability of gA to induce the H_{II} phase in diacyl-PC's with acyl chain lengths >16 has also been shown to depend on the $\beta^{6.3}$-channel conformation (Van Echteld et al., 1982; Killian & De Kruijff, 1986). Our results indicate that gA is able to induce a change in di-C_8-PC micelles; again, the change is dependent on the $\beta^{6.3}$-channel conformation.

Solubilization of gA by Di-C_6-PC. When gA is added to diacyl lipid dispersions with acyl chain lengths of ≤7, a negative CD spectrum is always observed, indicating that gA is not present in the channel conformation. We have found no conditions for $n \leq 7$ that will yield the "channel" conformation, presumably because the lipids are too short, and yet the gA is "soluble". Alone, gA is almost insoluble in water (<10 mg/L) (Wallace, 1990); however, when included in an aqueous dispersion of di-C_6-PC, gA becomes solubilized.

Previous ^{13}C NMR studies of gA added to MPPC micelles as a dry powder, where it is predominantly double-helical, have shown that in this conformation gA interacts very little with the hydrophobic core of the micelles; only after the channel conformation is achieved are the lipid acyl chains within the micelles significantly perturbed (Urry et al., 1979). Our results support the idea that double-helical gA molecules may be aggregated on the surface of the lipid micelles (Spisni et al., 1979; Masotti et al., 1980). ^1H NMR spectroscopy is sensitive to the structure and packing of phospholipids in model membrane systems (Roberts et al., 1978); the spectrum in Figure 6B indicates that the lipid organization is not altered when gA is present within the di-C_6-PC/gA dispersion. Nevertheless, there is no precipitate, and as gA itself is insoluble in water, the gA molecules must be associated in some manner with the di-C_6-PC particles, probably with their surfaces. The unchanged ^1H NMR spectrum of di-C_6-PC in the presence of gA may be due to the rapid motions of both components.

Electron microscopy of the di-C_6-PC/gA dispersions revealed that while the organization of the lipid particles appeared unchanged, aggregation of individual particles may have occurred.[3] The fraction of gA monomer present in the dispersions, based on SE-HPLC results, was found to be 0.38 and 0.60 for di-C_6-PC and di-C_7-PC, respectively. On the basis of the CD spectra (Figure 1), these are *not* $\beta^{6.3}$-helical

[3] Aggregation "on the grid" may not represent aggregation in solution, for the ^1H NMR resonances remain narrow (Figure 6B).

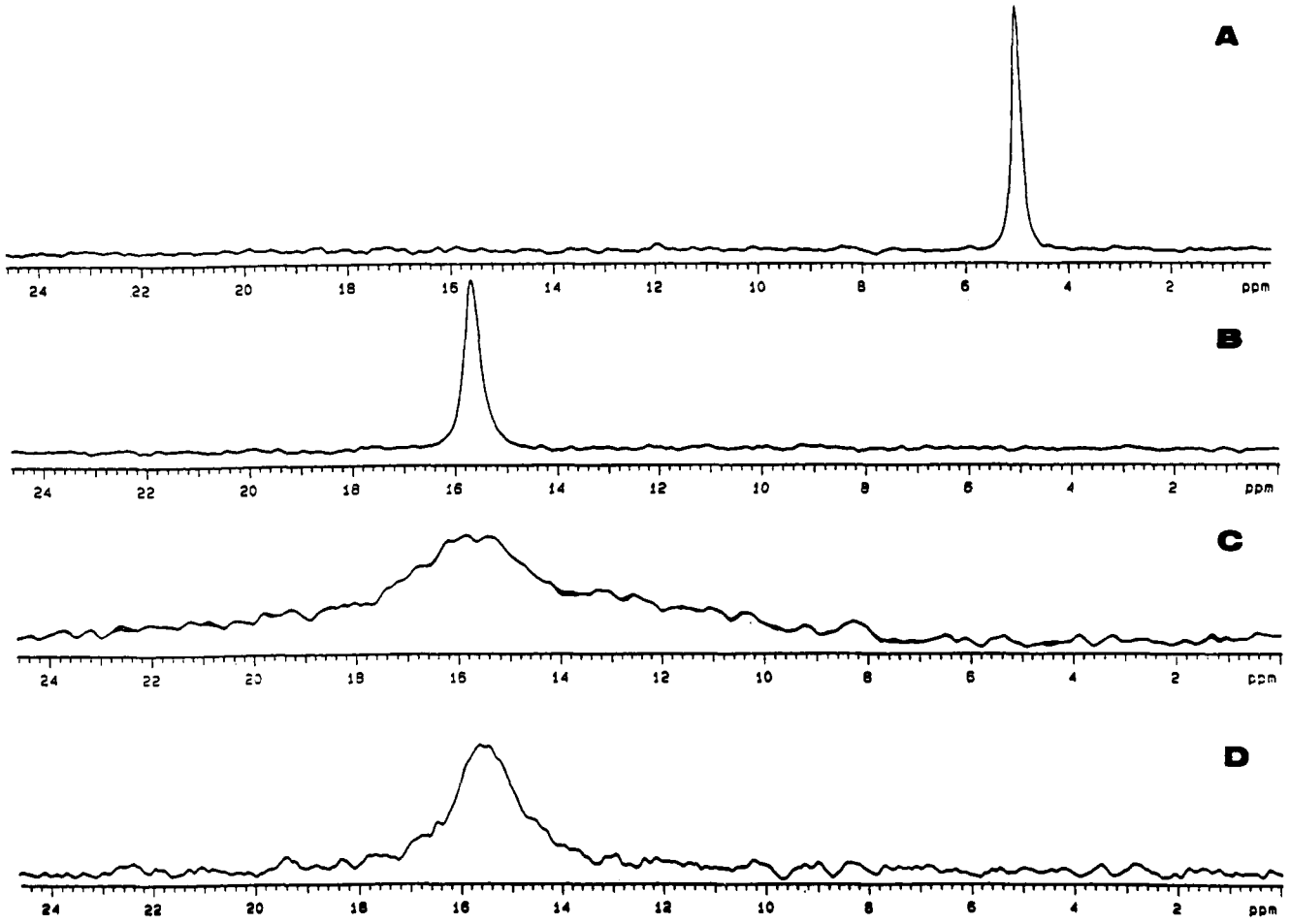

FIGURE 8: ^{31}P-NMR spectra of (A) di-C_8-PC, unsonicated; (B) di-C_8-PC, unsonicated with 10 mM Pr^{3+}; (C) gA/di-C_8-PC, unsonicated with 10 mM Pr^{3+}; and (D) gA/di-C_8-PC, sonicated with 10 mM Pr^{3+}. (Conditions as in Figure 6.)

monomers. These findings suggest that although both gA monomers and double-helical dimers associate with the surface of the short-chain lipid particles, gA is not able to penetrate the lipid or to form the $\beta^{6.3}$-helical configuration in di-C_6-PC or di-C_7-PC.

n = 8 Is the "Threshold" for the Channel Conformation. CD spectroscopy indicated that the channel conformation could be obtained when gA was codispersed with the di-C_8-PC; the results, however, depended on a number of sample preparation conditions. All traces of solvent had to be removed from the gA/lipid film prior to hydration. Insufficient drying resulted in an intermediate CD pattern, indicating that a mixture of both channel and nonchannel conformations was present. Increasing the gA:di-C_8-PC ratio to 1:14 also resulted in an intermediate CD spectrum. Heating, which has previously been shown to promote channel formation in longer chain lipids, again yielded an intermediate CD pattern for gA/di-C_8-PC. These results suggest that di-C_8-PC represents the minimal acyl chain length for the gA $\beta^{6.3}$-helical conformation.

Dependence of gA Conformation on Acyl Chain Length. Numerous models have been proposed which predict that "hydrophobic matching" occurs between the transmembrane segment of an integral membrane protein and the hydrocarbon chains of a lipid bilayer (Owicki et al., 1978; Engelman & Zaccai, 1980; Mouritsen & Bloom, 1984, 1993; Fattal & Ben-Shaul, 1993). The basic assumption of these models is that the more flexible lipid hydrocarbon chains will adapt to the thickness of the rigid, lipophilic core of a protein by stretching, squashing, and/or tilting. The lipid–protein interaction free energy (ΔF), which can be described as the sum of a surface (head-group region) term and a chain (hydrophobic core) term, is generally at a minimum when the length of the lipophilic region of the protein (d_P) matches that of the hydrophobic core of the lipid (d_L) (Fattal & Ben-Shaul, 1993). When $d_P > d_L$, the lipid acyl chains must stretch to match the length of the protein, resulting in a positive ΔF. For gramicidin, these effects may be further modulated by specific interactions between tryptophan rings and lipid head groups (O'Connell et al., 1990; Becker et al., 1991; Hu et al., 1993; Koeppe et al., 1994).

In support of these theories, the mean lifetime of gA channels increases as the bilayer thickness decreases (Hladky & Haydon, 1972; Elliott et al., 1983). In monoacylglycerol squalene bilayers, the lifetime increases as the lipid hydrocarbon thickness decreases betweeen 29 and 21.7 Å and then remains relatively constant, suggesting that the length of the lipophilic exterior of the gA channel is approximately 21.7 Å (Elliott et al., 1983). When the thickness of the membrane exceeds that of the gA channel, the membrane is thought to deform locally, or "dimple", to accommodate the channel (Huang, 1986; Helfrich & Jakobsson, 1990). Our results also support the concept of "hydrophobic matching". We find that the gA channel conformation is not attainable when the diacyl chain length is ≤ 7. This is probably due to an incompatibility between the lengths of the lipophilic region of the gA channel and a pair of lipid acyl chains, which for di-C_7-PC ranges from approximately 10 Å in the liquid-crystalline phase (Lewis & Engleman, 1983) to approximately 23 Å in the gel phase (Janiak et al., 1976). Given that Trp–head-group interactions would be required to stabilize the $\beta^{6.3}$ helices, the acyl chains of di-C_7-PC are simply not long enough

to accommodate the rest of gramicidin. (The tryptophans preclude the possibility that a gA channel could protrude beyond the lipid and be stable.)

For di-C_8-PC, in which acyl chains are slightly longer, the gA channel conformation is attainable. Upon incorporation of the gA channel into di-C_8-PC, the lipid acyl chains are expected to stretch into an extended conformation to accommodate the length of the channel. This could explain why the gA/di-C_8-PC dispersion is so dependent on experimental conditions. For example, when the gA/di-C_8-PC dispersion is heated at 68 °C, an intermediate CD spectrum is obtained (Figure 2B), indicating that a mixture of conformations, both single- and double-helical, is present. For di-C_{10}-PC which normally forms bilayers, the gA channel conformation is easily attained, suggesting that the channel length and acyl chain length are more compatible. In this situation, and in longer lipids, the $\beta^{6.3}$-channel-form gA is preferred because of favorable hydrogen bonding between Trp indoles and lipid head groups. (In any double-helical conformation, some of the Trp's would be buried.)

Effect of gA on Di-C_8-PC Organization. While both di-C_6-PC and di-C_7-PC form optically clear micellar dispersions in water, di-C_8-PC forms an unstable, turbid solution which readily separates into two layers just above its cmc (Tausk et al., 1974; De Haas et al., 1971). The high viscosity of the opaque lower lipid-rich phase suggests that di-C_8-PC probably forms large, disk-shaped or elongated cylindrical micelles (De Haas et al., 1971). The next higher homologue in the series, di-C_9-PC, is the first short-chain diacyl lipid to form bilayers (liposomes) in aqueous dispersions (De Haas et al., 1971). We found that the addition of gA to the di-C_8-PC dispersion results in a loss of phase-separation, indicating that the presence of gA has a stabilizing effect on the lipid aggregates. Furthermore, in the presence of gA, the di-C_8-PC has a more turbid, "milky" appearance, revealing possible aggregation or fusion (Taraschi et al., 1983) of the gA/lipid particles. It has recently been shown that gA induces aggregation and fusion of DOPC vesicles, resulting in an increase in vesicle size and a mixing of the aqueous contents (Tournois et al., 1990). Electron microscopy revealed a distinct change in the particle size and morphology upon inclusion of gA with di-C_8-PC, but not with di-C_6-PC. Although the staining and/or the plating method used to obtain the electron micrographs may have resulted in an overestimation of the particle sizes [alternate methods have reported smaller dimensions for the di-C_6-PC and di-C_8-PC lipid particles (Lin et al., 1986; Tausk et al., 1974)], the main conclusion is that the gA/di-C_8-PC particles are much enlarged relative to those of di-C_8-PC alone. We suspect that even larger particles would be observed in unsonicated samples, based on the broader ^{31}P resonances (cf. Figure 8C,D).

Both the ^1H and ^{31}P NMR spectra suggest that the lipid organization is affected by the inclusion of gA in the di-C_8-PC dispersions. The line broadening in both spectra suggests that the size of the aggregates has increased. All of the di-C_8-PC ^1H resonances—except the choline—are broadened (and some are shifted; Figure 6D), indicating that gA interacts with the entire lipid molecule. These spectral changes could be due to "bound and extended" lipid chains that exchange with more distant lipids.

The gA/di-C_8-PC dispersions exhibited a single ^{31}P resonance signal in both the absence and the presence of Pr^{3+}. The Pr^{3+} ion shifted the ^{31}P resonance downfield, indicating that all the phospholipid head groups remain accessible to the Pr^{3+} ion (the resonance is broadened by gA; Figure 8C,D). Had sealed vesicles been present, an additional upfield resonance signal would have been observed. Sonicating the dispersion (Figure 8D) also did not yield sealed vesicles.

There are several conditions that would explain the single resonance observed for the gA/di-C_8-PC dispersion with Pr^{3+}: (i) vesicles are present, but are unsealed or "leaky"; (ii) the mixed gA/lipid particles are bilayer–disks, lacking an internal space; or (iii) very large micelles are present. It has been suggested that "leaky" membranes can result either from a length mismatch between a lipid and an integrated peptide or from channels forming within aggregates of peptides (Deuticke et al., 1983). The incorporation of glycophorin into di-C_{18}-18:1_c/18:1_c-PC vesicles has been shown to cause the vesicles to become permeable to Dy^{3+} (Gerritsen et al., 1979). The barrier properties can be restored to the di-C_{18}/glycophorin vesicles by the addition of 10% 1-18:1_c-lyso-PC, suggesting that discontinuities in the bilayer between protein-perturbed and free phospholipid molecules might be responsible for the observed high permeability of the vesicles. The gA-induced changes in MPPC micelles result in the formation of "vesicle-like" structures, including some that are not well sealed (Spisni et al., 1983). Finally, it has recently been demonstrated that gA enhances the transbilayer movement of phospholipids ("flip") and induces nonspecific leaks in unilamellar DOPC vesicles and in human erythrocyte ghosts (Classen et al., 1987; Tournois et al., 1987, 1990). The diameter of such defects was estimated to range from 1 to 4 nm (Tournois et al., 1990). The $\beta^{6.3}$ conformation has been shown to be a prerequisite for both the "flip" enhancement and the nonspecific leakage (Tournois et al., 1988).[4]

It is also possible that the incorporation of gA as the $\beta^{6.3}$-helical channel might induce the formation of stable bilayer disks. The driving force for the formation of disks, rather than closed vesicles, is mainly entropic (Bian & Roberts, 1990). The micellelike di-C_8-PC lipid molecules would prevent the edges of the bilayer from contacting water, while the gA dimer would act as an anchor holding the bilayer together. This would result in an increase in the overall entropy of the system by allowing the smallest possible particles to form. Both melittin (Sessa et al., 1969) and bile salts (Muller, 1981) have been shown to stabilize bilayer disks or flat sheets of lipid bilayer. In the arrangement of a disk, all the lipid phosphate groups would be exposed to the exterior surface and would be available to interact with the Pr^{3+} cation.

The size changes in the sonicated gA/di-C_8 lipid aggregates were confirmed by electron microscopy, which revealed the presence of large (100–150 nm) vesicle-like structures, which have a tendency to align in rows. It is apparent that the morphology of the lipid particles has changed dramatically due to the incorporation of gA (Figure 7D). Taken together, the above findings all suggest that it is the conformation of gA upon interaction with the lipid, in particular the $\beta^{6.3}$ channel configuration, which is responsible for inducing the changes in the particle size and aggregational properties of di-C_8-PC.

Many vital membrane processes such as membrane fusion, the transbilayer movement of lipids, and protein insertion and translocation involve organizational changes in lipids. These processes are often modulated by amphiphilic and/or hydrophobic peptides and/or proteins. The results presented here provide further evidence for conformational coupling between intrinsic membrane proteins and their neighboring lipids, and emphasize the importance of protein tertiary structure for these interactions.

[4] Pr^{3+} could not go through the $\beta^{6.3}$ gA channel because the ion is trivalent, but the ion could "leak" around the outside due to disruption of the lipid by the peptide.

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