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Conformation of Gramicidin in Relation to Its Ability To Form Bilayers with Lysophosphatidylcholine†

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ABSTRACT: The ability of gramicidin to induce bilayer formation in lysophosphatidylcholine (LPC) systems was investigated as a function of the conformation of the peptide. The conformation was varied by using different solvents to cosolubilize gramicidin and lipid. Using circular dichroism (CD), it was found that when codissolved in trifluoroethanol (TFE), after drying and subsequent hydration, gramicidin is mainly present in the single-stranded $\beta^{6.3}$-helical configuration, whereas when using chloroform/methanol or ethanol as the solvent, it is proposed that the dominant conformation of gramicidin in the membrane is that of the double-stranded antiparallel dimer. Employing ^{31}P NMR, the stoichiometry for bilayer formation was found to be 6 to 7 lipid molecules per gramicidin monomer, when samples were prepared from TFE, whereas a stoichiometry of 4 was found when chloroform/methanol or ethanol was the solvent. Upon heating the latter samples, a conversion was observed in the CD pattern toward that indicative of the $\beta^{6.3}$-helical configuration. This change was accompanied by an increase in the extent of bilayer formation. Next, it was investigated whether the conformation of gramicidin and its ability to induce bilayer formation were dependent on the lipid acyl chain length. CD measurements of samples prepared from TFE indicated that gramicidin, independent of acyl chain length, was present in the $\beta^{6.3}$-helical configuration but the intensity of the ellipticities at 218 nm increased with the length of the acyl chain. The extent of bilayer formation in these samples was found to be largely chain length independent. In contrast, when ethanol was used as the solvent to codissolve peptide and lipid, the extent of bilayer formation increased with the chain length. CD measurements of these latter samples showed that the gramicidin molecules were now present in a dominantly "non-channel" configuration, most likely as an antiparallel dimer, and that again a chain length dependence was observed in the magnitude of the ellipticities at 218 nm. The results are discussed in terms of the possible mechanism of bilayer formation in dispersions of gramicidin and LPC in relation to the conformational behavior of the peptide.

Gramicidin is a linear, hydrophobic peptide that can form cation-selective transmembrane channels in model as well as in biological membranes [for review, see Andersen (1984) and Urry (1985a)]. It is produced by *Bacillus brevis* as a mixture of gramicidins A, B, and C in a molar ratio of about 80/5/15. The structure of gramicidin A is HCO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-NHCH$_2$CH$_2$OH. In gramicidins B and C, the tryptophan at position 11 is replaced by phenylalanine and tyrosine, respectively (Sarges & Wilkop, 1965a–c; Gross & Witkop, 1965).

In addition to its channel properties, the hydrophobic pentadecapeptide gramicidin also has a dramatic effect on lipid structure [for a review, see Killian and De Kruijff (1986)]. In bilayer-forming lipids, such as diacylphosphatidylcholines, gramicidin can induce H_{II} phase formation, provided that the lipid acyl chain length is longer than 16 carbon atoms (Van Echteld et al., 1982). For this effect, the tryptophan residues

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of gramicidin appeared to be essential (Killian et al., 1987). In addition, studies in which gramicidin was added from different organic solvents to preformed model membranes strongly suggested that the three-dimensional structure of the peptide is also important and that the $\beta^{6,3}$ configuration is responsible for the H_{II} phase inducing activity of gramicidin (Tournois et al., 1987). The lipid structure-modulating activity of gramicidin not only is evident in bilayer-forming diacyllipids but also is expressed in its interaction with lysophosphatidylcholine (LPC)[1] which has only one acyl chain; this lipid on its own prefers a micellar organization, but when mixed with gramicidin, it organizes in extended lamellar structures (Killian et al., 1983; Pasquali-Ronchetti et al., 1983). ^{2}H NMR studies on these systems, using selectively chain-deuteriated 16:0-LPC's, demonstrated a similar order profile along the acyl chain as for bilayers of pure DPPC in the liquid-crystalline phase (Killian et al., 1986). The ability of gramicidin to induce bilayer formation when mixed with LPC is particularly intriguing since LPC itself destabilizes membranes and is commonly used to solubilize membrane proteins. As occurs in the H_{II} phase inducing activity of gramicidin (Killian et al., 1987), also in its ability to form bilayers with LPC, the tryptophans of the peptide play an essential role. From studies with gramicidins A, B, and C and with tryptophan-formylated gramicidin, in which the indole NH protons of all four tryptophan residues are replaced by formyl groups, it appeared that the extent of bilayer formation occurred in the order of gramicidin A > gramicidin C ≥ gramicidin B > tryptophan-*N*-formylated gramicidin (Aranda et al., 1987). No significant differences were observed between the use of gramicidin A or the natural mixture of gramicidins. That the ability to induce bilayer formation is specific for gramicidin and derivatives and that it is not a common property of hydrophobic peptides are demonstrated by the observation that a hydrophobic α-helical pentadecapeptide, which consists of alanine and aminobutyric acid and is used as a model for the antibiotic peptide alamethicin (Jung et al., 1983), does not induce bilayer formation (Aranda et al., 1987).

In studies on the channel properties of gramicidin, it was reported that bilayer formation occurs on heat incubation of the peptide with lipid, when the gramicidin was added as a dry powder to egg LPC micelles (Pasquali-Ronchetti et al., 1983). In this lamellar complex, of which the lipids consist predominantly of 16:0-LPC and 18:0-LPC, a stoichiometry was found of 1 gramicidin molecule per 8–10 lipid molecules (Spisni et al., 1983). In contrast, ^{31}P NMR studies showed a 1 to 4 stoichiometry of peptide to lipid for bilayer formation of gramicidin with 16:0-LPC, when the dispersion was prepared by hydration of a mixed lipid/peptide film that had been dried from chloroform/methanol (1/1) (Killian et al., 1983; Aranda et al., 1987). Since differences in the conformation of gramicidin occur, depending on the method of sample preparation (Killian et al., 1988), the possibility should be considered that the reason for the different stoichiometries of the lamellar structures could be that gramicidin occurs in different conformations in the two systems.

This conformational postulate was addressed by determining the effect of the conformation of gramicidin on its bilayer-forming activity with LPC micelles. The conformation of the peptide was varied in two ways. First, by variation of the organic solvent in which gramicidin and the lipid were co-dissolved prior to dispersion, and second, by incubation of the samples at elevated temperatures. The conformation of gramicidin was monitored by circular dichroism, and bilayer formation was followed by ^{31}P NMR. These data indicate that it is the conformation of gramicidin on interaction with the lipid that determines the stoichiometry of bilayer formation with LPC and that the $\beta^{6,3}$-helical conformation of gramicidin is most effective in this respect.

For further insight into the role of the conformation of the peptide in relation to its interaction with LPC, gramicidin-induced bilayer formation was investigated by using lysophosphatidylcholines of varying acyl chain lengths. It will be shown that the conformation of gramicidin also plays a decisive role in determining the chain length dependence of bilayer formation. These results will be discussed from the perspective of the conformational behavior of the peptide and in terms of the molecular mechanism whereby bilayer formation is induced in mixtures of gramicidin and LPC.

Materials and Methods

Materials. Gramicidin was purchased from ICN Nutritional Biochemicals Corp. (Cleveland, OH) as the natural mixture of gramicidins A, B, and C (Sarges & Witkop, 1965a–c) and used without further purification. 1-Lauroyl-*sn*-glycero-3-phosphocholine (C12:0-LPC), 1-myristoyl-*sn*-glycero-3-phosphocholine (C14:0-LPC), 1-palmitoyl-*sn*-glycero-3-phosphocholine (C16:0-LPC), and 1-stearoyl-*sn*-glycero-3-phosphocholine (C18:0-LPC) were obtained from Avanti Biochemicals (Birmingham, AL). All other chemicals were of analytical grade.

Sample Preparation. Stock solutions of gramicidin and lipid were prepared in chloroform/methanol (1/1 v/v). For each experiment, a constant amount of 50 μmol of lipid was used, and the desired amount of gramicidin was added. The mixture was dried on a rotary evaporator and then dissolved in 1 mL of organic solvent. When ethanol was used, the gramicidin/lipid film was dissolved by heating for 1 min at 45 °C (temperature of the water bath) and/or by removing the solvent on the rotary evaporator and redissolving the sample in 1 mL of ethanol. This procedure was necessary to avoid solubility problems and to obtain a homogeneous peptide/lipid film. After 1-h incubation at room temperature, the solvent was removed, and the sample was stored overnight under high vacuum. The mixture was hydrated in 1.5 mL of 10 mM NaCl, and after 2-h incubation at 30 °C, the sample was transferred to a 10-mm NMR tube for ^{31}P NMR measurements. For ^{23}Na NMR and CD measurements, the sample was diluted with 10 mM NaCl to give an approximate 0.5 mM solution of gramicidin. Next, the sample was sonicated at 30 °C and centrifuged, as described elsewhere (Killian et al., 1988), and the supernatant was diluted to the desired final gramicidin concentration. The gramicidin content in the supernatant was determined by measurement of the absorbance after 30-fold dilution of the sample in methanol, using a molar extinction coefficient of 20 700 cm^{-1} M^{-1} at 280 nm (Killian et al., 1988), and the lipid content was determined by a phosphorus assay (Dittmer & Wells, 1979). In all samples, the gramicidin/LPC ratio in the supernatant was the same as the initial peptide/lipid ratio. Heat incubations were carried out at 68 °C using unsonicated dispersions.

Nuclear Magnetic Resonance. ^{31}P NMR measurements were performed at 30 °C on a Jeol FX-100 spectrometer operating at 40.26 MHz, using 4K data points, a 20-μs 90° pulse, a 15-kHz spectral width, and a 1-s interval between pulses. Continuous broad-band proton decoupling was applied with an input power of 15 W. Between 2000 and 5000 free

[1] Abbreviations: CD, circular dichroism; CSA, chemical shift anisotropy; LPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; PC, diacylphosphatidylcholine; T_1, longitudinal relaxation time; TFE, trifluoroethanol.

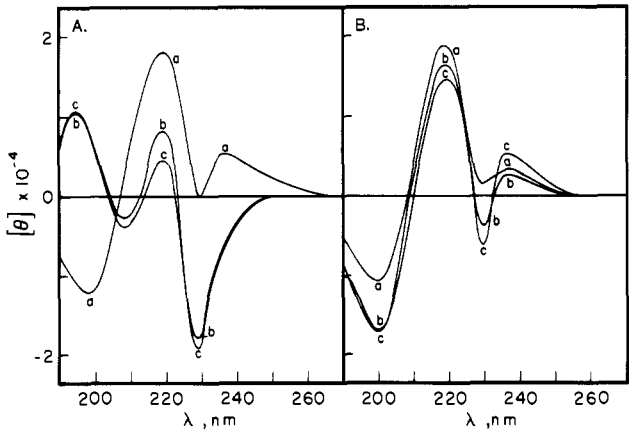

FIGURE 1: CD spectra of sonicated dispersions of gramicidin and 16:0-LPC in a 1/8 molar ratio of peptide to lipid before (A) and after 20 h of heat incubation at 68 °C (B). Samples were prepared by using TFE (a), chloroform/methanol (1/1 v/v) (b), and ethanol (c) to codissolve peptide and lipid. See text for details.

induction decays were accumulated to which, prior to Fourier transformation, an exponential multiplication was applied, resulting in a 30-Hz line broadening. The relative amount of bilayer signal was calculated by subtraction of the isotropic signal from the spectrum, followed by measurement of the area of both the remaining bilayer component and the total spectrum. The error was estimated to be about 5%.

^{23}Na NMR experiments were carried out as described elsewhere (Killian et al., 1988), using a gramicidin concentration of 0.25 mM in the presence of 10 mM NaCl.

CD Measurements. CD spectra were recorded at 30 °C with a Cary 60 spectropolarimeter as described previously (Masotti et al., 1980), using a 0.2-mm optical path-length cell and a gramicidin concentration of 0.20 mM.

RESULTS

Interaction of Gramicidin with 16:0-LPC. (A) Circular Dichroism. In order to test the hypothesis that different conformations of gramicidin are responsible for the differences in its bilayer-forming capacity on combination with LPC, first investigated was whether the conformation of the peptide can be controlled by drying the samples from different solvents as occurs for diacylphosphatidylcholines [see Killian et al. (1988)].

Figure 1A shows that indeed, as for diacyl-PC's, drying from TFE leads to a CD spectrum with positive peaks at 218 and 235 nm, a negative inflection at 229 nm, and negative ellipticity below 208 nm. This spectrum has been shown to be representative of the $\beta^{6.3}$-helical conformation of gramicidin (Urry, 1985a,b). When ethanol or chloroform/methanol is used, the CD characteristics are different; there now occur a large negative peak at 229 nm, a strong, positive band below 208 nm, and only a weak positive ellipticity near 220 nm. In these solvents, either all the peptide is present in a different conformation or a conformational heterogeneity exists with much less gramicidin present in the $\beta^{6.3}$-helical configuration. For methanol, similar spectra were obtained as for chloroform/methanol (data not shown).

(B) Phosphorus-31 NMR. ^{31}P NMR is a convenient technique with which to monitor the bilayer-inducing activity of gramicidin in LPC. When the lipids are organized in micelles, due to fast tumbling a complete motional averaging of the chemical shift anisotropy (CSA) will occur, resulting in one sharp isotropic signal. Upon formation of an extended lamellar phase, the only motional averaging of the CSA that occurs is due to a fast axial rotation of the lipids about their

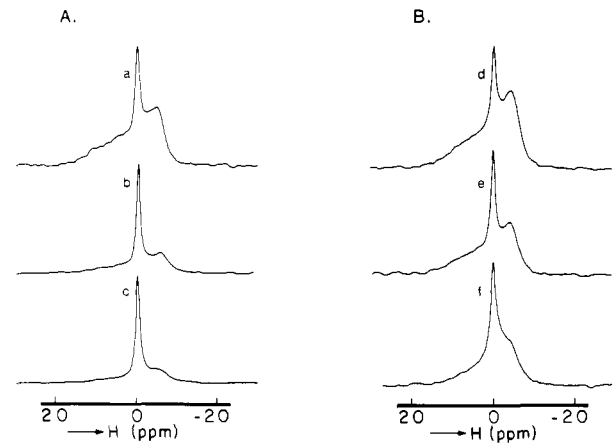

FIGURE 2: ^{31}P NMR spectra at 40.26 MHz of dispersions of gramicidin and 16:0-LPC in a 1/8 molar ratio of peptide to lipid before (A) and after 20 h of heat incubation at 68 °C (B). Samples were prepared from TFE (a and d), chloroform/methanol (1/1 v/v) (b and e), and ethanol (c and f). See text for details.

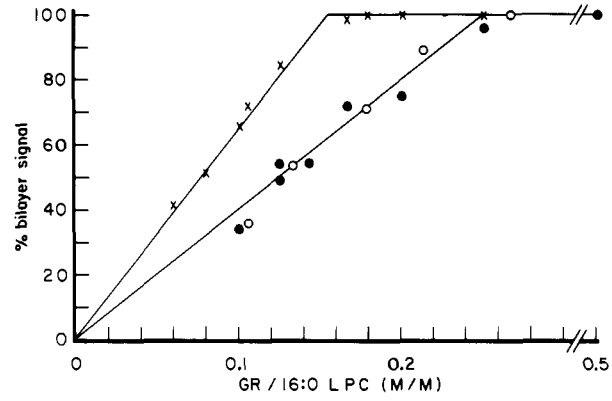

FIGURE 3: Percentage of bilayer signal as a function of the gramicidin/lipid ratio in samples prepared from TFE (×), chloroform/methanol (1/1 v/v) (○), and ethanol (●). See text for details.

long axis. This results in an axially symmetric ^{31}P NMR spectrum with a low-field shoulder and a high-field peak (Seelig, 1978) which are separated by 15–20 ppm in case of gramicidin/LPC mixtures (Killian et al., 1983, 1986). Figure 2A shows that, for gramicidin/16:0-LPC (1/8 molar ratio) samples and for each solvent used to codissolve peptide and lipid, the spectra are a superposition of a bilayer signal with a CSA of 15–20 ppm and an isotropic component. However, remarkable differences can be observed in the extent of bilayer formation. When the films are dried from TFE prior to dispersion, about 85% of the lipids give rise to a bilayer type of spectrum. For both ethanol and chloroform/methanol, this percentage is much lower (50% and 59% bilayer formation, respectively). In all three solvents, an approximately linear relationship is found between the extent of bilayer signal and the gramicidin content of the sample (Figure 3) up to a ratio of gramicidin to lipid above which 100% bilayer is formed. From these data, the stoichiometry is found to be 6–7 molecules of 16:0-LPC per gramicidin molecule when the samples are dried from TFE and about 4 lipid molecules per gramicidin molecule when the films were dried from chloroform/methanol and from ethanol. In conjunction with the CD data, these observations show that the conformation of gramicidin indeed is related to the bilayer-forming activity of the peptide and that in the $\beta^{6.3}$-helical configuration the bilayer-forming activity of gramicidin is most pronounced.

This is further demonstrated by the effect of heat incubation of the samples. As shown in Figure 1B, and similar to the

behavior of gramicidin in diacyllipids [see Killian et al. (1988)], heat treatment results in a change of the spectral characteristics in a direction toward the $\beta^{6.3}$-helix. This change is accompanied by an increase in the percentage of bilayer component in these samples (Figure 2B). The relative amounts of bilayer signal after heating were 88%, 80%, and 72% for TFE, chloroform/methanol, and ethanol, respectively. Similarly, it was found, using ^{31}P NMR, that bilayer formation occurred when gramicidin was added as a dry powder to a dispersion of LPC micelles and that the extent of bilayer formation increased with heat incubation (data not shown).

(*C*) *Sodium-23 NMR.* In the channel conformation, gramicidin has a strong interaction with Na ions, which can be followed by ^{23}Na NMR longitudinal (T_1) relaxation experiments (Urry et al., 1979; Urry, 1988). When Na ions in the bulk solution can exchange rapidly with sites in the gramicidin channel, a decrease in T_1 is observed. Indeed, the solvent dependence as well as the effect of heating on the conformation of gramicidin was reflected in the ^{23}Na NMR relaxation times and thus in the degree of interaction of the peptide with Na ions. In pure 16:0-LPC micelles in the presence of 10 mM NaCl, a longitudinal relaxation time was found of about 60 ms. This value of T_1 was markedly decreased to about 24 ms for a sample containing 0.25 mM gramicidin in the presence of 10 mM NaCl, when gramicidin was incorporated via cosolubilization of lipid and peptide (molar ratio 1/8) in TFE. When samples were initially dissolved in ethanol, a smaller reduction in the magnitude of T_1 was observed to about 37 ms, and the value decreased further upon heat incubation of the sample (data not shown), indicating heat-induced conversion of additional molecules of gramicidin to the $\beta^{6.3}$-helical conformation.

Conformation of Gramicidin in Relation to the Gramicidin/Lipid Ratio. When gramicidin/LPC mixtures are dried from different solvents, then, similar to the observations in diacyllipids (Killian et al., 1988), the conformation of gramicidin in the bilayer after removal of the solvent and hydration of the samples appears to be affected by the peptide/lipid ratio. This is shown in Figure 4 for samples with molar ratios of peptide to lipid of 1/4 and 1/10. For each of the solvents used, lowering the gramicidin/lipid ratio causes the spectra to change in a direction toward that of the $\beta^{6.3}$-helical configuration. Most likely, this is the result of differences in the conformation of gramicidin in the organic solvent, or it could reflect the occurrence of gramicidin/gramicidin contacts in the lamellar complex after hydration. If the conformational behavior in the solvent were responsible for the observed dependence of the CD pattern on the gramicidin/lipid ratio, then prolonged heat incubation can be expected to convert the CD pattern for all samples in a direction toward the $\beta^{6.3}$-helical configuration, with the final result being CD spectra which are independent of the gramicidin/lipid ratio. Accordingly, a heat incubation was carried out with the TFE samples. As is shown in Figure 4, even after extensive heating, a difference in the CD pattern can still be observed. Apparently, gramicidin/gramicidin interactions occurring in the bilayer at high gramicidin/lipid ratios are responsible for retention of this small difference, rather than conformational changes in the organic solvent.

Effect of Varying the Acyl Chain Length. Next investigated was the effect of varying the acyl chain length, using dispersions in which gramicidin and lipid were originally codissolved in either TFE or ethanol. As can be seen from Figure 5, remarkable differences are found between solvent systems. When the samples are dried from TFE, the ^{31}P NMR spectra

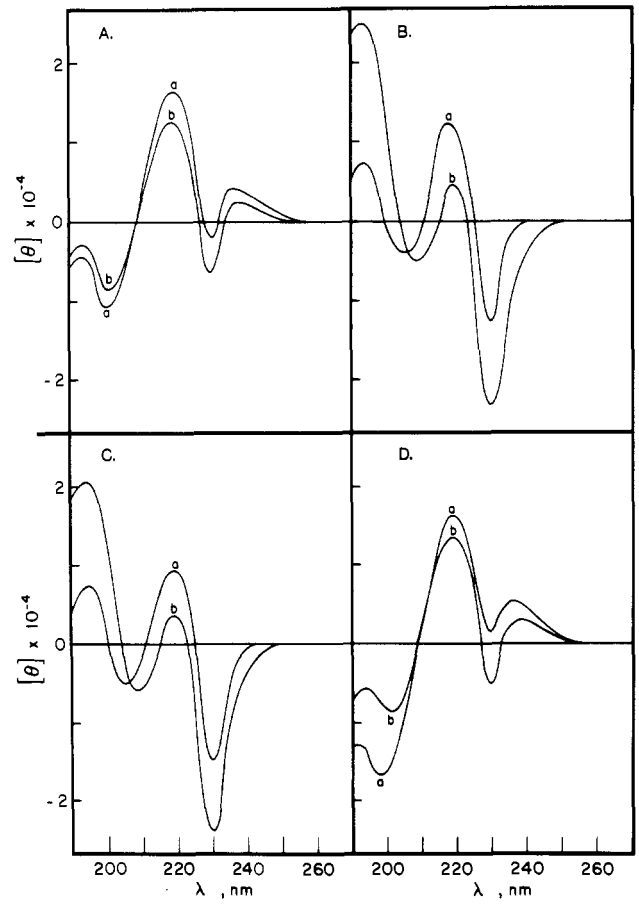

FIGURE 4: CD spectra of sonicated dispersions of gramicidin with 16:0-LPC in a 1/10 (a) and a 1/4 (b) molar ratio of peptide to lipid. Samples were prepared using TFE (A), chloroform/methanol (1/1 v/v) (B), and ethanol (C). (D) was obtained from the same dispersion as (A) but after 20-h heat incubation at 68 °C. See text for details.

are more similar for different acyl chain lengths than when they are dried from ethanol, where the extent of bilayer formation at a given gramicidin/lipid ratio is strongly dependent upon the acyl chain length (Figure 5B). In Figure 6, the percentage of bilayer signal is plotted as a function of the lipid acyl chain length for various gramicidin/lipid ratios. Recalling that, when TFE is used, the incorporated state is one gramicidin molecule to six or seven 16:0-LPC molecules (see Figure 3), in all cases in Figure 6A it is evident that the bilayer-inducing activity of gramicidin is higher and is not markedly affected by the lipid acyl chain length. In contrast, for samples dried from ethanol, the extent of bilayer formation is remarkably chain length dependent (Figure 6B). From the relationship between the percentage of bilayer signal and the gramicidin/lipid ratio, stoichiometries of bilayer formation can be calculated of 1.8, 3.4, and 4.0 LPC molecules per gramicidin monomer for 12:0-LPC, 14:0-LPC, and 16:0-LPC, respectively, with an estimated maximal error of 0.4. For 18:0-LPC, a value of 5.2 is obtained upon extrapolation of the percentage of bilayer formation induced at an initial molar ratio of 1/8 of peptide to lipid.

Figure 7A,C shows that, as for diacyllipids (Killian et al., 1988), the CD patterns of the gramicidin/LPC samples strongly depend upon the solvent from which they are prepared. When TFE is used, the CD spectra are characteristic of the $\beta^{6.3}$-helical configuration (Figure 7A), but when prepared from ethanol, the samples exhibit very different CD characteristics (Figure 7C). In general, small differences were noted in the magnitude of the ellipticity of the narrow extremum near 230 nm, which is due to the tryptophan side

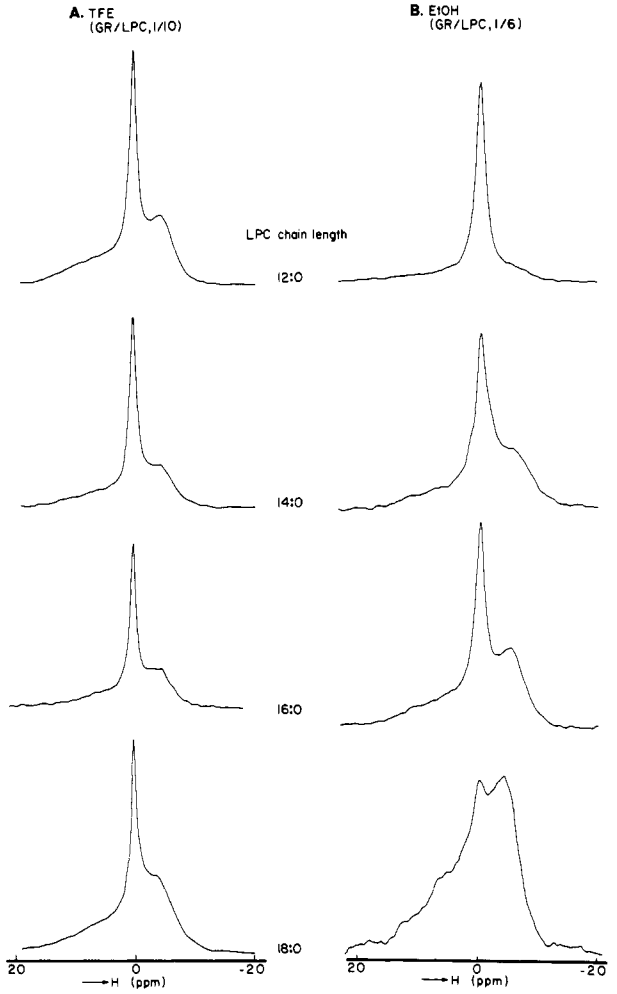

FIGURE 5: ^{31}P NMR spectra at 40.26 MHz of dispersions of gramicidin and LPC with varying chain length, *as indicated in the figure.* Samples were prepared from TFE (A) in a 1/10 molar ratio of peptide to lipid and from ethanol (B) in a 1/6 molar ratio of peptide to lipid. See text for details.

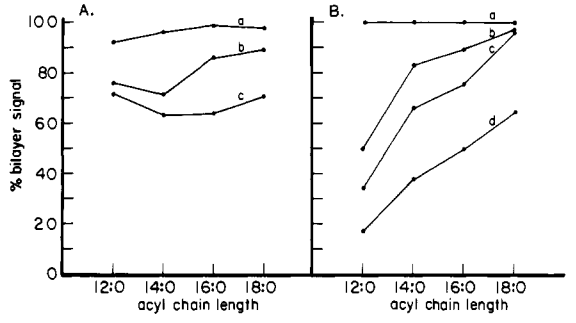

FIGURE 6: Percentage of bilayer signal as a function of the lipid acyl chain length for gramicidin/LPC dispersions prepared from TFE (A) in molar ratios of peptide to lipid of 1/6 (a), 1/8 (b), and 1/10 (c) and from ethanol (B) in molar ratios of peptide to lipid of 1/2 (a), 1/4 (b), 1/6 (c), and 1/8 (d). See text for details.

chains (Urry et al., 1975). Since these differences did not appear to be chain length dependent, at least not in a systematic way, and since they tended to disappear upon heat incubation (compare, for instance, the CD spectrum of gramicidin in 14:0-LPC in Figure 7A,B), it is likely that they can be ascribed to differences in the conformation of gramicidin in the organic solvent before or upon drying. However, for both solvent systems, a systematic increase is observed in the intensity of the band at 218 nm with the acyl chain length (Figure 7A,C), which is still present after heating (Figure 7B).

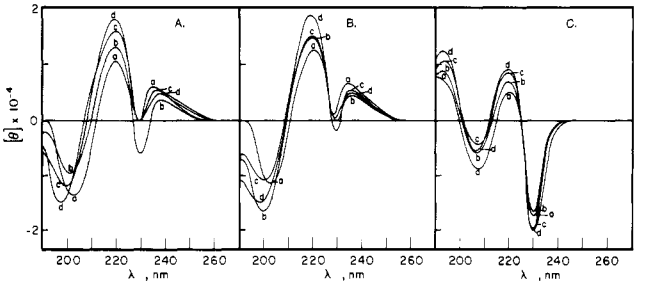

FIGURE 7: CD spectra of sonicated dispersions of (1/8 molar ratio) mixtures of gramicidin and LPC with varying acyl chain length [12:0-LPC (a), 14:0-LPC (b), 16:0-LPC (c), and 18:0-LPC (d)]. Samples were prepared from TFE (A) and ethanol (C). (B) was obtained from the same dispersion as (A) but after 20-h heat incubation at 68 °C. See text for details.

The ellipticity at 218 nm is considered to arise dominantly from the peptide carbonyl n–π^* transitions (Urry et al., 1975), and, therefore, it is most likely that these systematic differences are due to the peptide backbone.

Discussion

The above results clearly show that gramicidin can adopt different conformations in bilayers with LPC and that the nature of the interaction between gramicidin and the lipid is determined by the conformation of the peptide. Furthermore, as in the case of diacyl-PC's (Killian et al., 1988), the conformation in the membrane appears to be determined by the conformational behavior of gramicidin in the organic solvent from which samples are prepared. When peptide/lipid films are dried from TFE, it can be concluded from the CD data that, as with diacyl-PC's (Killian et al., 1988), gramicidin in its final configuration in the lamellar phase after hydration is dominantly present as a $\beta^{6.3}$-helix. When samples are dried from ethanol or chloroform/methanol, the CD patterns indicate that gramicidin is now present in another conformation. In analogy with the observed solvent dependence of the conformational behavior of gramicidin in diacyl-PC's (Killian et al., 1988), it is proposed that this conformation corresponds to an antiparallel double-stranded helix, because this is the dominant conformation of gramicidin in ethanol (Veatch et al., 1974; Arseniev et al., 1984) and at high concentrations in methanol (Fossel et al., 1974). The dimensions of the antiparallel double-stranded helix have been reported to be approximately the same as those of the single-stranded N–N dimeric channel (Wallace, 1986).

The differences in conformation of gramicidin in the various systems appear to result in distinct differences in the ability of the peptide to induce bilayer formation with LPC. A clear correlation was found between the extent to which the CD patterns demonstrate the presence of gramicidin in a $\beta^{6.3}$-helical configuration and the extent of bilayer formation, as derived from ^{31}P NMR data. This correlation was observed not only with a preparation of samples from different organic solvents but also after heat incubation of the samples. Previously similar differences in stoichiometry of bilayer formation were reported on preparation of a mixed gramicidin/LPC film from chloroform/methanol (1/1, by volume) (Killian et al., 1983; Aranda et al., 1987) and on external addition of gramicidin as a dry powder to egg LPC micelles, followed by heat incubation, during which gramicidin can be fully converted to its channel configuration (Pasquali-Ronchetti et al., 1983). From the results presented in this paper, it is clear that these differences in stoichiometry of bilayer formation can primarily be attributed to conformational differences of the peptide in the two systems.

Not only do the conformational differences lead to variations in the stoichiometry, but they also result in a different interaction with LPC's of varying acyl chain lengths. In samples prepared from TFE, the stoichiometry of bilayer formation appears to be rather independent of chain length (Figures 5A and 6A), whereas a preparation of samples from ethanol demonstrates a strong chain length dependence with the stoichiometry of bilayer formation increasing with the number of carbon atoms in the lipid acyl chain (Figures 5B and 6B).

The results suggest that two different mechanisms of bilayer formation occur: one mechanism in which gramicidin is mainly present in the $\beta^{6.3}$-helical configuration and in which the stoichiometry in the lamellar phase is about 1/7 (gramicidin/LPC, molar ratio) and where the value is nearly chain length independent. This ^{31}P NMR derived stoichiometry is in fair agreement with the stoichiometry of 1/8 to 1/10 reported on the basis of sucrose density gradient centrifugation experiments for mixtures of gramicidin and egg LPC in which the peptide was added externally as a dry powder to a solution of LPC micelles and incorporated in its channel configuration by prolonged heat treatment (Pasquali-Ronchetti et al., 1983; Spisni et al., 1983). In the second mechanism of gramicidin-induced bilayer formation in mixtures with LPC, the peptide is here proposed to be mainly present as an antiparallel dimer. The stoichiometry of bilayer formation, on the basis of ^{31}P NMR measurements, is now clearly acyl chain length dependent and is about 1/4 (molar ratio of peptide to lipid) for 16:0-LPC.

Previously, two mechanisms have been proposed for the mode of action of bilayer formation induced by gramicidin in LPC systems. First, a comparison was made with mixtures of LPC and free fatty acid, which form bilayers in a 1/1 stoichiometry (Jain et al., 1980), and it was proposed that the size and shape of the gramicidin channel may be considered equivalent to the volume occupied by free fatty acids when mixed with LPC (Pasquali-Ronchetti et al., 1983). Such a mechanism allows that the length of the gramicidin in relation to the thickness of the hydrophobic part of the bilayer plays a significant role and could explain the chain length dependence of the stoichiometry of bilayer formation as observed in samples dried from ethanol. In this mechanism, however, the situation could be more complex and involve lipid volume distribution instead of simply pairing acyl chain length with length of gramicidin structure [see Spisni et al. (1983)].

Another proposal for the mechanism of bilayer formation in gramicidin/LPC systems has been based on the shape/structure relationship as postulated for lipid phase behavior [see Tilcock (1986)]. According to this concept, the overall cone shape of LPC is responsible for its tendency to organize in micelles. Since in the $\beta^{6.3}$-helical configuration, gramicidin too could be considered to have a cone shape, due to the location of the four bulky tryptophan residues all near the C-terminus of the molecule, mixing of LPC and gramicidin in such a way that their shapes are complementary would result in the formation of an overall cylindrical complex, which according to the shape/structure relationship is predicted to prefer organization as bilayers (Killian et al., 1983; Brasseur et al., 1986). Since the complementarity of both shapes would not be affected as much by the chain length of the lipid, such a mechanism could in principle be compatible with the apparent lack of chain length dependence as observed upon preparation of samples from TFE. However, the mechanism has one major problem: it requires an orientation of the gramicidin molecule in the membrane with its N-terminus at the lipid/water interface, which is opposite to the channel configuration of the peptide (Urry et al., 1983) in which the N-termini are linked together by hydrogen bonding in the hydrophobic interior of the membrane and in which the C-termini are located near the lipid/water interface.

An alternative possibility to explain the different mechanisms of bilayer formation in gramicidin/LPC systems is based upon the tendency of gramicidin to self-associate. It has been suggested that gramicidin aggregation plays a role in channel functioning (Spisni et al., 1983; Stark et al., 1986) and in the induction of H_{II} phase formation (Brasseur et al., 1987; Killian et al., 1987). Moreover, aggregation has been proposed to occur in dispersions of gramicidin and LPC (Spisni et al., 1983; Killian et al., 1986) and is supported by electron microscopic, freeze–fracture studies (Spisni et al., 1983). It is quite possible that the aggregational behavior of gramicidin is dependent upon the conformation of the peptide and that, for a given conformation, it in addition may depend upon the acyl chain length. Since aggregation of gramicidin is expected to affect the interaction with LPC, it may thus be of importance in determining the extent of bilayer formation as well as in determining whether or not the stoichiometry is affected by the length of the acyl chain. In this context, it is interesting to note that the higher stoichiometry of bilayer formation for gramicidin as compared to tryptophan-*N*-formylated gramicidin was ascribed to a difference in conformational and/or aggregational behavior of the two peptides (Aranda et al., 1987).

As a general conclusion, the present study shows that, depending on its conformation, gramicidin can interact differently with lipids and, in general, it demonstrates the importance of knowledge about the conformation of peptides in order to understand peptide/lipid interactions.

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Registry No. C12:0-LPC, 20559-18-6; C14:0-LPC, 20559-16-4; C16:0-LPC, 17364-16-8; C18:0-LPC, 19420-57-6; gramicidin, 1405-97-6.

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Inhibition of Arginine Aminopeptidase by Bestatin and Arphamenine Analogues. Evidence for a New Mode of Binding to Aminopeptidases†

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ABSTRACT: The synthesis and inhibition kinetics of a new, potent inhibitor of arginine aminopeptidase (aminopeptidase B; EC 3.4.11.6) are reported. The inhibitor is a reduced isostere of bestatin in which the amide carbonyl is replaced by the methylene ($-CH_2-$) moiety. Analysis of the inhibition of arginine aminopeptidase by this inhibitor according to the method of Lineweaver and Burk yields an unusual noncompetitive double-reciprocal plot. The replot of the slopes versus [inhibitor] is linear (K_{is} = 66 nM), but the replot of the y intercepts ($1/V$) versus [inhibitor] is hyperbolic (K_{ii} = 10 nM, K_{id} = 17 nM). These results provide evidence for a kinetic mechanism in which the inhibitor binds to the S_1' and S_2' subsites on the enzyme, not the S_1 and S_1' subsites occupied by dipeptide substrates. Furthermore, structure–activity data for a series of ketomethylene dipeptide isosteres in which the amide ($-CONH-$) of a dipeptide is replaced with the ketomethylene ($-COCH_2-$) moiety show that the S_1 and S_1' subsites preferentially bind basic and aromatic side chains, respectively. These results are in agreement with the known substrate specificity of arginine aminopeptidase. The structure–activity data for several bestatin analogues, however, show that these compounds do not bind to the S_1 and S_1' sites of arginine aminopeptidase. A comparison of the data provides evidence that bestatin inhibits arginine aminopeptidase and possibly other aminopeptidases by binding to the S_1' and S_2' sites of the enzyme.

Aminopeptidases are hydrolytic enzymes that catalyze the removal of the amino-terminal residue from a peptide chain. The inhibition of aminopeptidases has been of interest since the isolation of bestatin **1** (Figure 1) (Umezawa et al., 1976a; Suda et al., 1976), a natural product found in culture filtrates of *Streptomyces olivoreticuli*, which potently inhibits aminopeptidase enzymes from a variety of sources. Bestatin has also been reported to have therapeutically useful effects both in vivo and in vitro (Umezawa et al., 1976b; Umezawa, 1984; Shimamura et al., 1984). Our studies of aminopeptidases have involved not only investigations into the mechanism of inhibition of these enzymes by bestatin but also synthetic studies toward the preparation of new inhibitors of aminopeptidases.

Our studies of one particular aminopeptidase, arginine aminopeptidase[1] [EC 3.4.11.6; L-Arginyl (L-Lysyl)peptide hy-

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