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Solvent History Dependence of Gramicidin-Lipid Interactions: A Raman and Infrared Spectroscopic Study

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ABSTRACT We have investigated the interactions between gramicidin and a model membrane composed of one phospholipid, dimyristoylphosphatidylcholine, as a function of the cosolubilization solvent and incubation time used in the sample preparation. Three organic solvents have been used; trifluoroethanol, a mixture of methanol/chloroform (1:1 v/v), and ethanol. Using Fourier transform infrared spectroscopy, we have demonstrated that the conformation adopted by gramicidin in the membrane is dependent upon the cosolubilization solvent used, and, only with trifluoroethanol, it is possible to incorporate gramicidin entirely as a $\beta^{6.3}$-helix. Moreover, Raman spectroscopy results indicate that the orientation of the tryptophan side chains in gramicidin and their interaction with the hydrocarbon chains and the carbonyl groups of the lipids are also dependent on the cosolubilization solvent. On the other hand, the effect of the incorporation of gramicidin on the thermotropism of the lipid bilayer was found to be dependent upon the conformation of gramicidin in the lipid bilayers.

INTRODUCTION

Gramicidin (80% gramicidin A, 5% gramicidin B, and 15% gramicidin C) is a hydrophobic linear peptide, which can form transmembrane channels that induce permeability to monovalent cations in biological membranes (Urry, 1984). In the channel conformation, gramicidin is considered to exist as a single-stranded right-handed $\beta^{6.3}$-helix (Urry et al., 1971; Nicholson and Cross, 1989; Prosser et al., 1991). Gramicidin is isolated from *Bacillus brevis* and is composed of 15 amino acids. 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}-$NH_2CH_2CH_2OH$. In gramicidin B and C, the tryptophan at position 11 is replaced by phenylalanine and tyrosine residues, respectively.

Gramicidin has been widely used as a model for the hydrophobic part of intrinsic membrane proteins. Recently, it has been shown by circular dichroism (Killian et al., 1988a), NMR spectroscopy (LoGrasso et al., 1988; Killian et al., 1988b), and high performance liquid chromatography (Bañó et al., 1991) that gramicidin can adopt various conformational states in hydrated phospholipid bilayers. A number of factors may determine the conformation that the gramicidin molecule ultimately adopts in a phospholipid dispersion, such as the peptide/lipid ratio, the solvent used to cosolubilize the phospholipid and gramicidin, the incubation time, and temperature (Killian et al., 1988a; Killian, 1992; Wallace, 1983; LoGrasso et al., 1988). Trifluoroethanol was originally suggested as a good cosolubilization solvent because of the monomeric nature of gramicidin in trifluoroethanol (TFE) solution (Urry et al., 1972). In fact, it has been shown that when gramicidin is added to diacylphosphatidylcholine model membranes from a solution in TFE, it is directly incorporated in the $\beta^{6.3}$ conformation (Tournois et al., 1987). By contrast, when gramicidin is incorporated from solvents such as chloroform or ethanol, in which it tends to form various intertwined dimers, the channel structure is slow to appear (Killian et al., 1988a; LoGrasso et al., 1988).

Vibrational spectroscopy is well-suited for the study of lipid-protein interactions, since it allows the investigation of the conformation of phospholipid molecules at different levels in the lipid bilayers and to follow structural changes that occur during the gel to liquid-crystalline phase transition. Moreover, it provides direct information on the conformation of proteins in lipid bilayers. A number of groups have investigated the interation between gramicidin and phospholipid bilayers using infrared spectroscopy (Cortijo et al., 1982; Lee et al., 1984; Naik and Krimm, 1986a,b; Davies et al., 1990). However, the methods of sample preparation used in these studies varied significantly. Therefore, in view of the solvent dependence of gramicidin conformation in model membranes, we have investigated in the present study the interaction between gramicidin and a model membrane composed of one phospholipid, dimyristoylphosphatidylcholine (DMPC), as a function of the cosolubilization solvent and the incubation time. Three organic solvents have been used: TFE, a mixture of methanol and chloroform (1:1 v/v), and ethanol.

By use of Fourier transform infrared spectroscopy (FTIR), we were able to demonstrate that the conformation adopted by gramicidin in the membrane is dependent upon the cosolubilization solvent, and, only with TFE, it is possible to incorporate gramicidin entirely as a $\beta^{6.3}$-helix. Moreover, in view of the fact that the four tryptophan residues of gramicidin are essential for channel formation (Killian and de Kruijff, 1986; O'Connell et al., 1990; Scarlata, 1988, 1991; Becker et al., 1991), we have used Raman spectroscopy to obtain detailed molecular information on the orientation and

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the environment of the tryptophan side chains in gramicidin (Hirakawa et al., 1978; Miura et al., 1988; Takeuchi et al., 1990) as well as on and their interactions with the hydrocarbon chains and the carbonyl groups of the lipids. On the other hand, the effect of the incorporation of gramicidin on the thermotropism of the lipid bilayer was found to be dependent on the cosolubilization solvent and the incubation time used in the sample preparation. Our results therefore clearly demonstrate the importance of the method of sample preparation in the study of lipid-protein interactions.

EXPERIMENTAL PROCEDURES

Materials

Gramicidin and DMPC were obtained from Sigma Chemical Co. (St. Louis, MO) and used without any further purification. Trifluoroethanol was purchased from Aldrich Chemical Co. (Milwaukee, WI) and methanol and chloroform from Fisher Scientific (Pittsburgh, PA). The salts used in the preparation of the buffers were of analytical grade.

Preparation of samples

Samples of DMPC/gramicidin were prepared in a 10:1 molar ratio by co-dissolving appropriate amounts of peptide and lipid in the organic solvent (1 ml). In order to obtain homogeneous peptide/lipid films, the samples were incubated at 52°C for 1 or 4 h and shaken on a vortex mixer at least a few times during the incubation cycle. After the incubation, the organic solvents were evaporated with a nitrogen stream followed by high vacuum overnight to ensure complete evaporation of the solvents. The samples were then hydrated with a Na_2HPO_4 buffer (100 mM) at pH 7.0, prepared in D_2O, and submitted to several cycles of heating (52°C)-vortex shaking-cooling (20°C). For the study of the phosphate region, the samples were hydrated with a HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) buffer (100 mM) at pH 7.0 prepared in H_2O.

FTIR measurements

Infrared spectra were recorded with a Bomem DA3-02 Fourier transform spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. Samples were inserted between CaF_2 or BaF_2 windows (Wilmad Glass Co. Inc, Buena, NJ) using 12-μm Mylar spacers. 250 interferograms were recorded with a resolution of 2 cm^{-1}, and each spectrum was corrected for the water contribution by subtracting appropriate polynomial functions. The spectra in the carbonyl region were deconvolved using the deconvolution software of Spectra-Calc (Galactic Industries, Salem, NH) which uses the deconvolution technique of Griffiths and Pariente with a narrowing parameter (γ) of 2.18 and an apodization filter of 0.25 (Griffiths and Pariente, 1986).

For polarized ATR measurements, we used an ATR unit (model TMP-220; Harrick Scientific Co., Ossining, NY) and a parallelogram germanium ATR crystal (50 × 20 × 2 mm, $\theta = 45°$) (Wilmad Glass Co. Inc., Buena, NJ). The crystal was cleaned in a plasma cleaner sterilizer (model PDC-3x G; Harrick Scientific Co.) prior to utilization. Oriented films of DMPC/gramicidin were prepared by spreading the aqueous sample on the germanium crystal. A Teflon bar was moved slowly back and forth along the surface until complete evaporation of the solvent. 250 interferograms were recorded with a 2-cm^{-1} resolution. The rotating wire-grid polarizer (Specac, UK) was placed in the sample compartment and was under manual control. All spectral data were analyzed with the SpectraCalc software.

Dichroic ratios (R) of the infrared bands were obtained by ratioing either the integrated intensities or the peak heights of the bands measured with the incident light polarized parallel and perpendicular with respect to the plane of incidence (Fringeli and Günthard, 1981; Hübner and Mantsch, 1991). If a uniaxial fiber-type distribution is assumed with respect to the normal of the ATR crystal (i.e., normal to the bilayer in the film), the order parameters $f(\theta)$ relating the orientation of the fiber axis and the normal of the ATR plate were calculated with the following equation (Hübner and Mantsch, 1991):

$$f(\theta) = \frac{R - 2}{R + 1.45} \cdot \frac{2}{3\cos^2\gamma - 1},$$

where γ is the angle between the transition moment of a given vibration and the fiber axis. The equation used is for thick films (Fringeli and Günthard, 1981), since the film thickness obtained with our sample preparation method is between 2.5–4 μm, which is much greater than the penetration depth of the evanescent wave (0.2–0.8 μm in the frequency range 4,000–1,000 cm^{-1} for germanium with $\zeta = 45°$) (Hübner and Mantsch, 1991). For the lipid hydrocarbon chains, the transition moments of the symmetric CH_2 stretching mode is assumed to be uniformaly distributed with an angle γ of 90°, while the angle γ was set to 0° for the carbonyl stretching vibration of the lipid head group. On the other hand, according to Nabedryk et al. (1982), the value of γ for the amide 1 band of gramicidin can be estimated to 22.6°. The mean angle between the lipid or peptide axis and the bilayer normal was then calculated from:

$$f(\theta) = \frac{3\cos^2\theta - 1}{2}.$$

Raman measurements

Raman spectra were recorded on a microcomputer-controlled Spex Model 1400 double monochromator (Savoie et al., 1979) with a multichannel detector (CCD9000 system from Photometrics Ltd., 1152 × 298 pixels EEV detector) using the 514.5-nm exciting line from a Spectra Physics Model 2020 argon ion laser. The power of the laser at the sample was about 250 mW, and all spectra were obtained with a spectral resolution of 2 cm^{-1}. Capillaries containing the samples were placed in a thermoelectrically regulated sample holder (Pézolet et al., 1983). By using emission lines of neon, the frequency calibration was achieve with an accuracy of 1 cm^{-1}. The entrance slit was 2 cm^{-1}, and the acquisition time was 5 min (an average of 10 acquisitions for each spectrum). Spectral manipulations were done using the SpectraCalc software, and a four-point Fourier interpolation was performed on all spectra.

RESULTS AND DISCUSSION

Conformational behavior of gramicidin in model membranes

FTIR spectroscopy is very useful for determining the conformations of peptides in lipid bilayers by analyzing the amide I region (Mendelsohn and Mantsch, 1986; Pézolet and Dousseau, 1990; Surewicz and Manstch, 1988). We have investigated the conformational behavior of gramicidin as a function of the solvent from which the DMPC/gramicidin samples were prepared by observing the frequency and the shape of the amide I band at approximately 1632 cm^{-1} (Urry, 1984).

Fig. 1 shows the infrared spectra of the amide I region for DMPC/gramicidin samples obtained at a temperature of 40°C (above the gel to liquid-crystalline phase transition temperature) for an incubation time of 1 h and as a function of the cosolubilization solvent. Similar results were obtained over the temperature range from 2° to 50°C and for an incubation time of 4 h. When the cosolubilization solvent is trifluoroethanol, the amide I band is sharp and located at 1632 cm^{-1}. This frequency has been shown to correspond to a $\beta^{6.3}$-helix which is the active conformation of the trans-

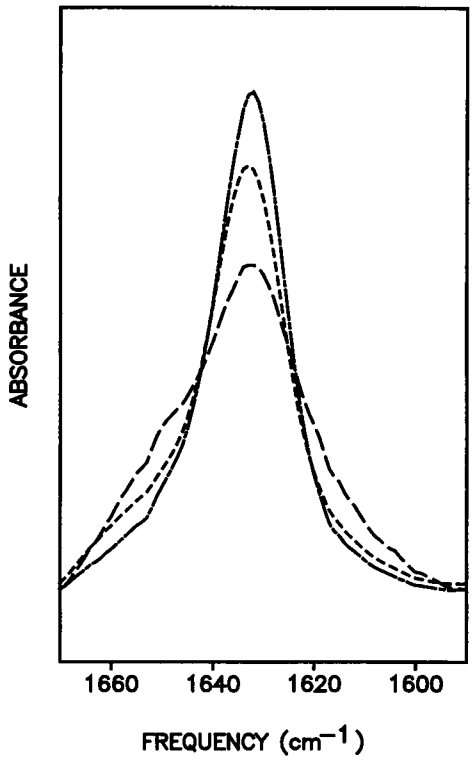

FIGURE 1 Infrared spectra at 40°C of the amide I region of gramicidin in DMPC/gramicidin systems incubated for 1 h and initially prepared in trifluoroethanol (— - —), a mixture of methanol/chloroform (1:1, v/v) (- - - -), and ethanol (— — —).

membrane channel (Urry, 1972). This result is in good agreement with the conformation observed by circular dichroism (Killian et al., 1988a). When a mixture of methanol/chloroform (1:1 v/v) or ethanol are used as the initial cosolubilization solvent, we observe a broadening of the amide I band and the frequency shifts toward slightly higher frequencies. These observations indicate that gramicidin, when incorporated in the lipid bilayer from a mixture of methanol/chloroform (1:1, v/v) or from ethanol adopts a different conformation (or possibly more than one conformation) compared to that obtained with trifluoroethanol.

These results are in agreement with those obtained by circular dichroism (Killian et al., 1988a), NMR spectroscopy (LoGrasso et al., 1988; Killian et al., 1988b), and high performance liquid chromatography (Bañó et al., 1991) that have shown that the active conformation of gramicidin, the $\beta^{6.3}$-helix (Urry et al., 1972), is favored when trifluoroethanol is used as the cosolubilization solvent (Killian et al., 1988a; LoGrasso et al., 1988; Killian et al., 1988b; Bañó et al., 1991). On the other hand, when a mixture of methanol/chloroform (1:1, v/v) or ethanol are used, other conformations like the double-stranded helix are known to be present. Our results therefore confirm that the conformation of gramicidin incorporated in lipid bilayers is dependent upon the cosolubilization solvent used in the sample preparation. Moreover, they also clearly demonstrate the potential of FTIR spectroscopy in determining structural changes in a peptide incorporated into a lipid bilayer.

Structure and environment of gramicidin side chains

Raman spectroscopy is useful in studying the structure of peptides incorporated into lipid bilayers. In particular, this method can provide information on the secondary structure of the peptide as well as on the structure and environment of the amino acid side chains (Takeuchi et al., 1990). In the present study, we have used Raman spectroscopy to investigate the structure and conformation of the tryptophan side chains in the peptide, as well as the structure of the tyrosine residue at position 11 in gramicidin C (present in a proportion of 15% in the mixture of gramicidins). The four tryptophan side chains of gramicidin are located in the carboxyl-terminal half of the peptide. In the channel conformation, tryptophan (15) is accessible to solvent molecules, tryptophan (9) is well buried in the membrane and for the two remaining tryptophan (11 and 13), it has be shown that their exposure to solvent molecules is dependent upon the thickness of the bilayer (Takeuchi et al., 1990).

More specifically, the 880-, 1340-, 1360-, 1550-cm^{-1} bands of tryptophan and the 830- and 860-cm^{-1} tyrosine doublet, have been used in the determination of the structure of gramicidin side chains in the lipid bilayers as a function of the cosolubilization solvents. For the tryptophan residues, each band detected is an overlap of the band due to all four tryptophan side chains present in gramicidin. The doublet at 1340 and 1360 cm^{-1} is a marker of the hydrophobicity of the environment of the indole ring (Harada et al., 1986; Miura et al., 1988), the frequency of the 880-cm^{-1} band reflects the strength of hydrogen bonding at the NH site of the indole ring (Miura et al., 1988), while the band at 1550 cm^{-1} gives the absolute value of the Cα-Cβ-C3-C2 torsional angle of tryptophan ($\chi^{2,1}$) (Miura et al., 1989). The tyrosine doublet at 830–860 cm^{-1} was used to observe the state of hydrogen bonding of the tyrosine residue (Kitagawa et al., 1979).

The 800–900-cm^{-1} spectral region is shown in Fig. 2. It is first possible to observe changes in the frequency of the 880-cm^{-1} band of the tryptophan residues. This band gives information on the strength of hydrogen bonding of the indole ring (Miura et al., 1988). When the cosolubilization solvent is trifluoroethanol, the frequency of the band is 873 cm^{-1}, and the band shifts to 874 and 875 cm^{-1} when methanol/chloroform or ethanol are used. The low frequency of these bands indicates that the NH sites of the four indole rings are strongly hydrogen-bonded (Miura et al., 1988) and that the strength does not vary significantly as a function of the cosolubilization solvent.

Since the samples used in the present study were prepared with gramicidin D, which is composed of 15% of gramicidin C in which the tryptophan at position 11 is replaced by a tyrosine residue, it was also possible to observe the vibrations of the tyrosine doublet in the 830–860-cm^{-1} spectral region. When gramicidin is initially incorporated into the lipid bilayers with trifluoroethanol, the tyrosine vibrations can be detected, but when the cosolubilization solvent is methanol/chloroform (1:1, v/v) or ethanol, the tyrosine bands are not

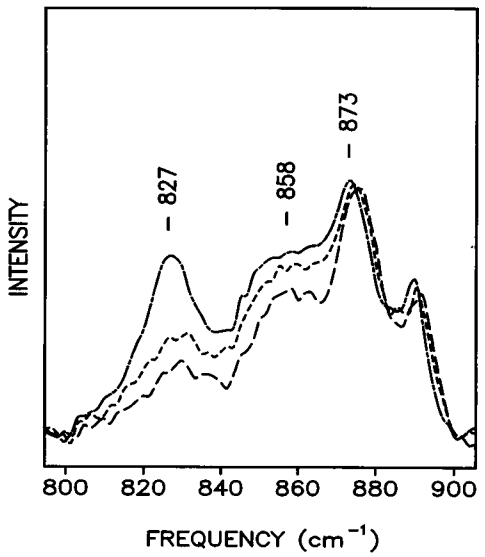

FIGURE 2 Raman spectra at 10°C of the 800–900 cm^{-1} region of DMPC/gramicidin incubated for 4 h and initially prepared in trifluoroethanol (— - —), a mixture of methanol/chloroform (1:1, v/v) (- - - -), and ethanol (— — —).

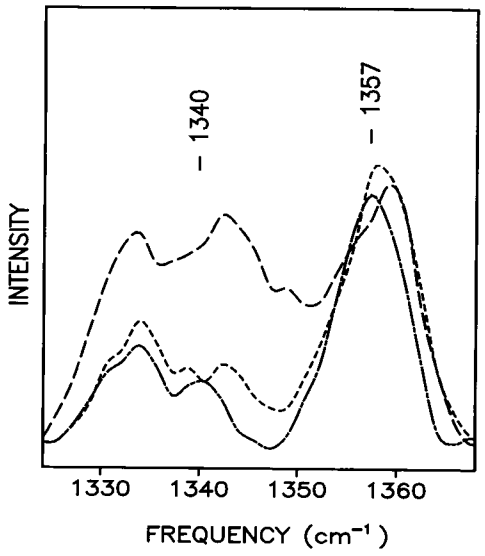

FIGURE 3 Raman spectra at 10°C of the 1340–1360 cm^{-1} region of DMPC/gramicidin incubated for 4 h and initially prepared in trifluoroethanol (— - —), a mixture of methanol/chloroform (1:1, v/v) (- - - -), and ethanol (— — —).

well resolved. The observed change in the intensity ratio of the two components of the doublet, $I(860\ cm^{-1})/I(830\ cm^{-1})$, suggests the formation of a strong hydrogen bond as the codissolution solvent changes from methanol/chloroform or ethanol to trifluoroethanol (Kitagawa et al., 1979).

The results obtained from the tyrosine doublet at 830–860 cm^{-1} and the tryptophan band at 880 cm^{-1} therefore indicate that the tryptophan NH sites and the tyrosine OH group are involved in the formation of strong hydrogen bonds, this effect being particularly pronounced in the system prepared from trifluoroethanol This result is in agreement with previous studies suggesting that the NH sites of the indole ring of tryptophan are pointing toward the lipid carbonyl groups (Ketchem et al., 1993; Woolf and Roux, 1993) and that hydrogen bonding between the tryptophan NH group and the lipid carbonyl groups are important for the stabilization of the $\beta^{6.3}$ conformation of gramicidin and for channel formation (O'Connell et al., 1990; Scarlata, 1988, 1991; Becker et al., 1991).

The relative intensities of the two components of the doublet at 1340–1360 cm^{-1} (Fig. 3) suggest differences in the environment of the tryptophan residues as the cosolubilization solvent changes from trifluoroethanol or the mixture of methanol/chloroform to ethanol. The intensity ratio, $I(1360\ cm^{-1})/I(1340\ cm^{-1})$, is near one when the cosolubilization solvent is ethanol, which indicates that most of the tryptophan residues are located in a hydrophobic environment but that the interactions with the acyl chains of the lipid are not very strong (Harada et al., 1986; Miura et al., 1988). In contrast, when trifluoroethanol or methanol/chloroform are used, the intensity ratio raises well above unity, which indicates that most of the tryptophan residues are well buried in the membrane, inaccessible to solvent molecules but most importantly, interacting strongly with the lipid molecules. The changes observed in the doublet at 1340–1360 cm^{-1} support the results obtained for the tyrosine doublet at 830–860 cm^{-1} and the tryptophan band at 880 cm^{-1}, which suggested the formation of stronger hydrogen bonds between the lipid carbonyl groups and the tryptophan NH sites when trifluoroethanol is used as the cosolubilization solvent.

Another spectral change is noticed in the frequency of the 1550 cm^{-1} band as a function of the cosolubilization solvent. It has been shown that the frequency of this band gives the absolute value of the torsional angle of the Cα-Cβ-C3-C2 linkage $\chi^{2,1}$ (Miura et al., 1989). When the cosolubilization solvent is trifluoroethanol, the frequency is 1548 cm^{-1} but when methanol/chloroform and ethanol are used as cosolubilization solvents, the frequency of the band increases to 1550 and 1553 cm^{-1}, respectively, as shown in Fig. 4. Angles associated with these frequencies are between 90° and 99° (Takeuchi et al., 1990). The $\chi^{2,1}$ angles obtained by Raman spectroscopy therefore clearly indicate that the tryptophan residues have different orientations depending on the cosolubilization solvent initially used in the sample preparation.

Effect of the incorporation of gramicidin on the lipid bilayers

Lipid acyl chain region

So far, the conformation of gramicidin in lipid bilayers has been investigated as a function of the cosolubilization solvent and incubation time. We have also investigated the effects of the incorporation of gramicidin on the acyl chains and interfacial regions of the lipid bilayers. Fig. 5 shows the acyl chain C-H stretching mode region for pure DMPC (*solid line*) and the complex between DMPC and gramicidin prepared from trifluoroethanol (*dashed line*)

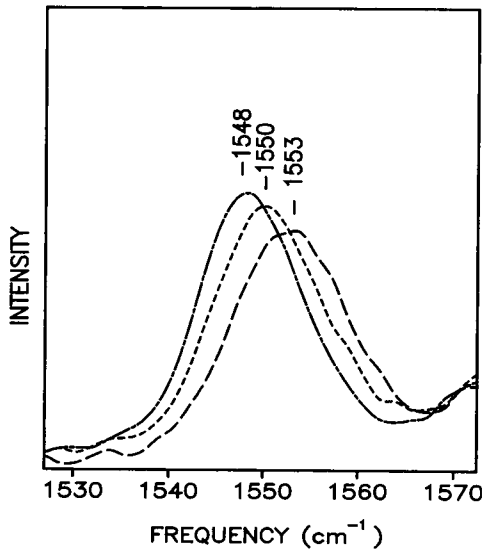

FIGURE 4 Raman spectra at 10°C of the 1550 cm^{-1} band of DMPC/gramicidin incubated for 4 h and initially prepared in trifluoroethanol (— - —), a mixture of methanol/chloroform (1:1, v/v) (- - - -), and ethanol (— — —).

below and above the gel to liquid-crystalline phase transition temperature of the lipid. This spectral region is dominated by two strong bands at 2920 et 2850 cm^{-1}, assigned to the methylene antisymmetric and symmetric stretching modes, respectively (Casal and Mantsch, 1984). Weaker band due to the asymmetric and symmetric stretching modes of the terminal methyl group are also observed near 2950 and 2870 cm^{-1}, respectively (Casal and Mantsch, 1984). The two methylene bands exhibit the same behavior as the temperature is increased: they become broader and they shift to higher frequency. The increase in bandwidth has been assigned to the increase of the rotational mobility of the acyl chains, while the frequency shift is due to the introduction of gauche conformers in the lipid acyl chains (Asher and Levin, 1977).

We have investigated the effect of the incorporation of gramicidin into the DMPC bilayers as a function of the incubation time for three different cosolubilization solvents, trifluoroethanol, a mixture of chloroform and methanol (1:1, v/v), and ethanol. Fig. 6 shows the temperature profiles derived from the frequency on the symmetric methylene stretching mode band near 2850 cm^{-1} for incubation times of 1 (*top*) and 4 h (*bottom*), respectively. The 2850 cm^{-1} feature was used instead of the 2917 cm^{-1} band, since it is less affected by the spectral contribution of the protein. For the lipid-protein systems prepared from the three cosolubilization solvents, with no regards of the incubation time, insertion of gramicidin results in an increase of the frequency of the 2850-cm^{-1} band, both below and above the gel to liquid-crystalline phase transition temperature of the lipid. This indicates that the interaction of gramicidin with DMPC bilayers leads to an increase in gauche conformers as a result of an increase in motional freedom of the lipid acyl chains (Dluhy et al., 1984; Casal and Mantsch, 1984). Moreover, the incorporation of gramicidin induces changes in the gel to liquid-crystalline phase transition temperature of the lipid and decreases the phase transition cooperativity. These results are in agreement with previous FTIR studies that indicated that gramicidin introduces disorder in the lipid acyl chains (Lee et al., 1984; Naik and Crim, 1986a, b; Cortijo et al., 1982).

Differences in the thermotropic behavior of DMPC/gramicidin bilayers are also observed as a function of the cosolubilization solvents, these differences being more pronounced when the incubation time is 1 h. More

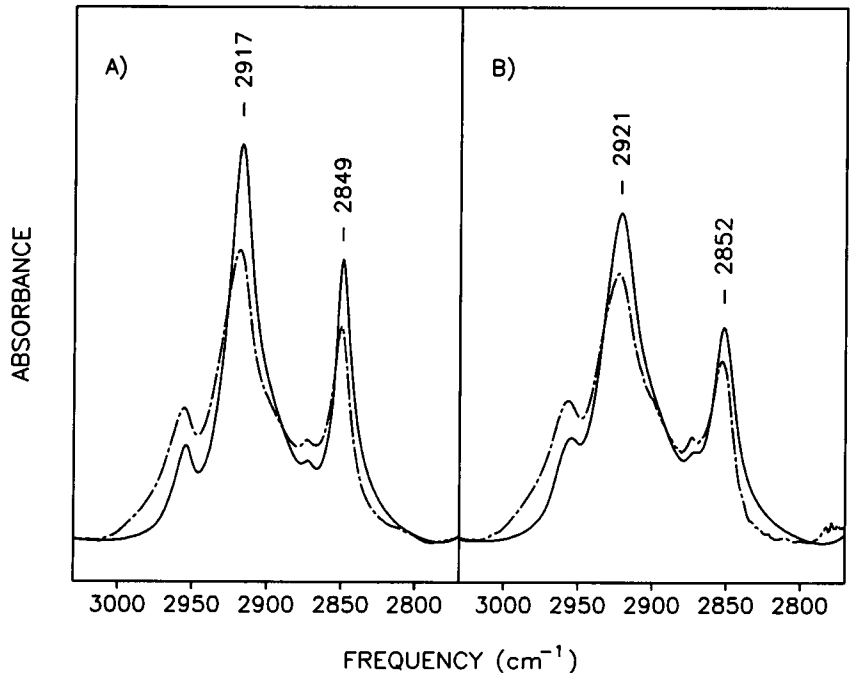

FIGURE 5 Infrared spectra in the C-H stretching mode region of DMPC (*solid line*) and of DMPC/gramicidin initially prepared in trifluoroethanol and incubated 4 h (*broken line*) in the gel (10°C) (*A*) and liquid crystalline (50°C) (*B*) phases.

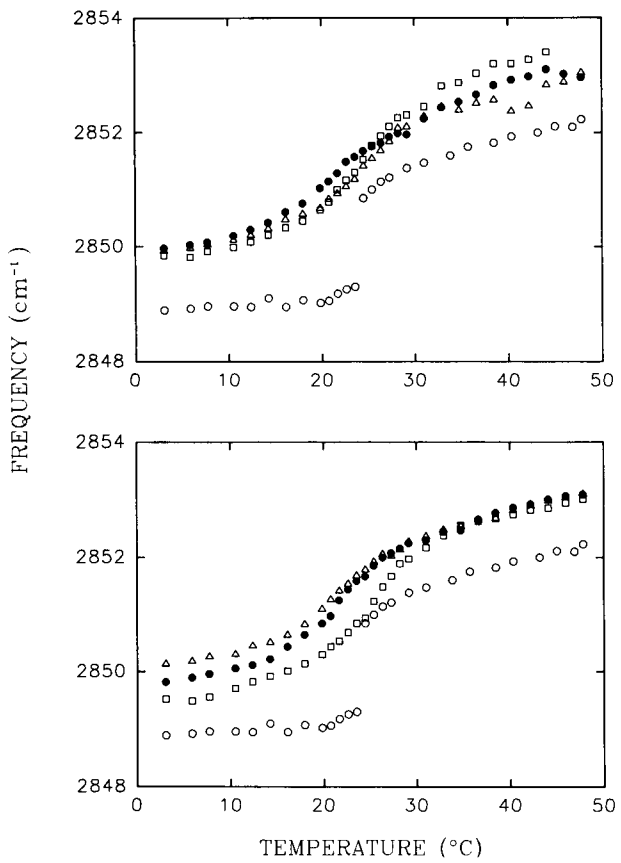

FIGURE 6 Temperature dependence of the frequency of the CH_2 symmetric stretching vibration in DMPC and DMPC/gramicidin (10:1 molar ratio) (○), initially prepared in trifluoethanol (●), a mixture of methanol/chloroform (1:1, v/v) (△), and ethanol (□), for incubation times of 1 h (*top*) and 4 h (*bottom*).

specifically, a marked decrease in the phase transition cooperativity is observed when trifluoroethanol is used as a cosolubilization solvent. This suggests a stronger effect of the incorporation of gramicidin on the lipid acyl chains. When the incubation time is increased to 4 h, the phase transition temperature and cooperativity of the systems prepared from trifluoroethanol and methanol/chloroform (1:1, v/v) are similar. These results indicate that the effect of gramicidin on the thermotropism of DMPC bilayers is very sensitive to several parameters influencing the conformation of the peptide in the membrane (*vide supra*). More specifically, our results demonstrate that the effect of gramicidin is greater when the peptide is incorporated as a $\beta^{6.3}$-helix and decreases with the presence of other conformations. The solvent dependence of the gramicidin conformation in lipid bilayers can also explain the differences observed as a function of the incubation time, these differences being more easily observable when the incubation time is 1 h. When the incubation time is increased to 4 h, the conformation of gramicidin tends toward the $\beta^{6.3}$-helix even if the cosolubilization solvent initially used for the cosolubilization does not favor the active conformation of the peptide (Killian et al., 1988a).

Carbonyl stretching mode region

The effect of gramicidin on the interfacial region of DMPC bilayers has been investigated from the carbonyl stretching mode region of the infrared spectra. In general, for hydrated samples, the carbonyl stretching mode region (1680–1750 cm^{-1}) consists of two bands originating from the two ester carbonyl groups in the molecule. These bands are more clearly distinguishable after resolution enhancement by Fourier deconvolution (Cameron et al., 1982). It has been shown that the band at about 1740 cm^{-1} is associated with phospholipid carbonyl groups that are not hydrogen-bonded to water while the low frequency band at about 1725 cm^{-1} is assigned to hydrogen-bonded C=O groups (Blume et al., 1988).

Fig. 7 shows the carbonyl stretching mode region of the original and deconvolved infrared spectra of DMPC dispersions in the absence and presence of gramicidin as a function of the solvent initially used to cosolubilize the protein and the lipid. The results are shown for the systems in the gel phase (10°C) and for an incubation time of 1 h, but similar trends were also observed in the liquid-crystalline phase. We can observe that the effect of gramicidin on the interfacial region of the lipid bilayer is more pronounced as the cosolubilization solvent changes from ethanol to methanol/chloroform (1:1, v/v) to trifluoroethanol. This effect is reflected by the difference in the relative intensities of the two components of the carbonyl band compared to those observed for pure DMPC, the high-frequency band becoming slightly more intense in the presence of gramicidin for the systems prepared from trifluoroethanol or methanol/chloroform (1:1, v/v). This indicates a decrease in the number of carbonyl groups that are hydrogen-bonded to water molecules. This could be possibly due to a slight change in the conformation of the interfacial region of the lipid molecule as a function of the conformation of the peptide in the bilayer, the carbonyl groups becoming less exposed to the water molecules. Another possibility is hydrogen bonding of the lipid carbonyl groups with amino acid side chains of the gramicidin molecules. The later is supported by recent solid-state NMR spectroscopy results that have shown that the NH groups of the indole ring of tryptophans are pointing toward the lipid carbonyl groups (Ketchem et al., 1993). This conclusion is also supported by our Raman spectroscopy results which suggest that a fraction of tryptophan side chains in the gramicidin molecules are hydrogen-bonded. Moreover, fluorescence spectroscopy results have shown that hydrogen bonding between the tryptophan NH groups and the lipid carbonyl groups are important for the stabilization of the $\beta^{6.3}$ conformation of gramicidin and for channel formation (O'Connell et al., 1990; Scarlata, 1988, 1991; Becker et al., 1991).

Phosphate stretching mode region

The effect of gramicidin on the polar head group of DMPC has been investigated from the phosphate stretching mode region (between 1000 and 1300 cm^{-1}) of the infrared spectra. The results (not shown) indicate that there is no change in this

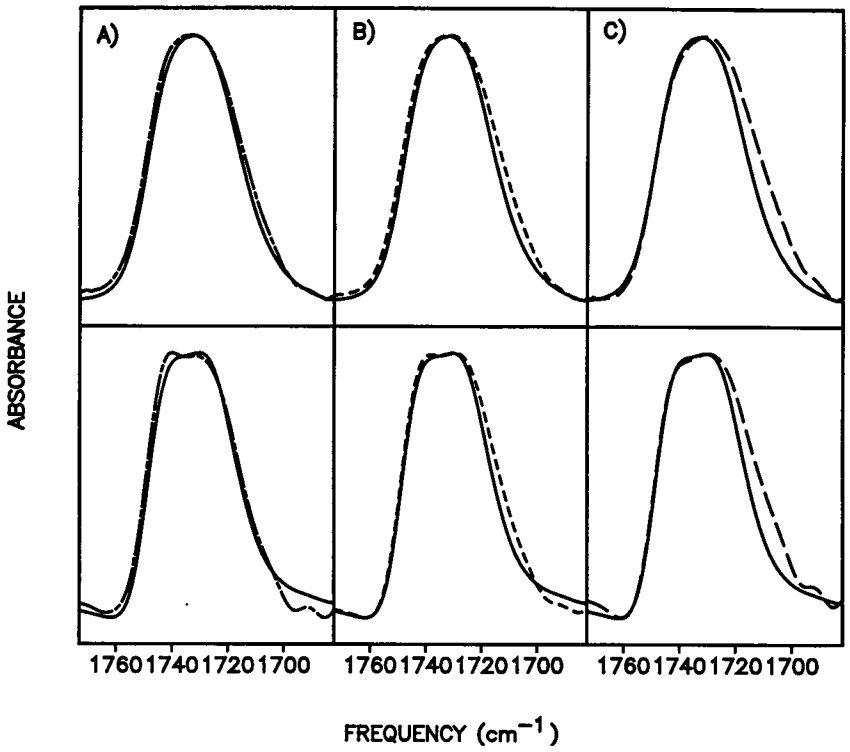

FIGURE 7 Original (*top*) and Fourier deconvolved infrared spectra (*bottom*) at 10°C of the carbonyl stretching mode region of (*solid line*) DMPC and DMPC/gramicidin initially prepared in trifluorethanol (*A*, — - —), a mixture of methanol/chloroform (1:1, v/v) (*B*, - - - -), and ethanol and incubated for 1 h (*C*, — — —).

spectral region for DMPC in the absence and presence of gramicidin, with no regards of the cosolubilization solvent or incubation time. This result is not surprising, since the gramicidin molecule is known to incorporate in the bilayer and therefore have very little effect on the lipid head group.

Orientation measurements

In order to obtain information on the orientation of the peptide and lipid components of DMPC/gramicidin complexes as a function of the cosolubilization solvent and incubation time, polarized infrared attenuated total reflectance spectra of oriented films of DMPC in the absence and presence of gramicidin were recorded. We have investigated the orientation of gramicidin and DMPC in systems of DMPC/gramicidin as a function of the cosolubilization solvent and the incubation time. Table 1 is a comparison of the dichroic ratios (R) and the mean angles θ calculated as described under Materials and Methods. All these value are the average of at least three independent measurements. The orientation of different groups in pure DMPC bilayers are in agreement with those reported in previous studies (Hübner and Mantsch., 1991; Okamura et al., 1986).

In the gramicidin-lipid systems, we observe that there is a reorientation of the lipid molecules compared to the orientation obtained in the pure lipid system, the average orientation of the lipid acyl chain changing from about 25° in the pure lipid system to values between 30° and 35° in gramicidin-lipid bilayers. This effect is observed for incubation times of both 1 and 4 h. It should be noted that the

TABLE 1 Dichroic ratio (r) and calculated angle (θ) for selected spectral absorption bands of DMPC and DMPC/gramicidin oriented films

Samples	Spectral bands					
	ν_{CH_2} sym		$\nu_{C=O}$		$\nu_{amide\ I}$	
	r	θ	r	θ	r	θ
DMPC	1.07 ± 0.03	25° ± 2°	1.31 ± 0.06	66° ± 2°		
DMPC/gramicidin incubation time of 1 h						
Trifluoroethanol	1.19 ± 0.02	31° ± 1°	1.49 ± 0.04	62° ± 1°	3.3 ± 0.2	42° ± 2°
Methanol/chloroform	1.22 ± 0.05	31° ± 2°	1.53 ± 0.05	62° ± 1°	3.0 ± 0.3	44° ± 3°
Ethanol	1.18 ± 0.02	30° ± 1°	1.48 ± 0.05	62° ± 1°	3.2 ± 0.2	42° ± 2°
DMPC/gramicidin incubation time of 4 h						
Trifluoroethanol	1.23 ± 0.02	32° ± 1°	1.51 ± 0.08	62° ± 2°	3.0 ± 0.2	44° ± 2°
Methanol/chloroform	1.26 ± 0.05	33° ± 2°	1.59 ± 0.09	61° ± 2°	2.6 ± 0.2	47° ± 3°
Ethanol	1.31 ± 0.05	35° ± 2°	1.55 ± 0.08	61° ± 2°	2.6 ± 0.2	47° ± 3°

change of orientation observed with the incorporation of gramicidin could also be interpreted as a broadening of the orientation distribution. This would be in agreement with the results obtained from the frequency of the symmetric methylene stretching mode band which indicates that the incorporation of gramicidin increases the number of gauche conformers in the lipid acyl chains (*vide supra*). The average orientation of the peptide molecule is ranging from 42° to 47°. This indicates that the peptide is not exactly aligned with the lipid molecules in solid lipid films (Okamura et al., 1986). However, the orientation of both the lipid and the peptide molecule in the gramicidin-lipid system does not appear to be significantly dependent on the cosolubilization solvent or the incubation time used in the preparation of the samples.

CONCLUSIONS

Infrared and Raman spectroscopic techniques have been used in the present study to investigate the solvent dependence of the interaction between the peptide gramicidin and model lipid bilayers. The results clearly indicate a change in the secondary structure of gramicidin as a function of the cosolubilization solvent initially used to prepare the peptide-lipid system. More specifically, a $\beta^{6.3}$-helix is obtained when trifluoroethanol is used as the cosolubilization solvent and a mixture of conformations are observed when ethanol of chloroform/methanol (1:1, v/v) are used. Moreover, Raman spectroscopy results indicate that the environment and orientation of the tryptophan side chains in gramicidin are dependent upon the cosolubilization solvent. The change in the conformation of the gramicidin molecule is also reflected in the effect of the peptide on the thermotropism of the lipid acyl chains and on the formation of hydrogen bonds between the lipid carbonyl groups and the NH sites of the tryptophan side chains in the peptide.

The differences observed in the gramicidin-lipid system as a function of the cosolublization solvent and the incubation time clearly illustrate the importance of the method of sample preparation in the study of lipid-protein interactions. Moreover, the present study demonstrates the use of vibrational spectroscopy in studying lipid-protein interactions by allowing the observation of the effect of the peptide on the lipid bilayers as well as the conformation of the peptide in the same system.

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