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Deuterium Nuclear Magnetic Resonance Investigation of the Exchangeable Sites on Gramicidin A and Gramicidin S in Multilamellar Vesicles of Dipalmitoylphosphatidylcholine†

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ABSTRACT: Solid gramicidin A and S and their interaction with DPPC bilayers were examined by ^2H NMR as well as ^{31}P NMR and differential scanning calorimetry (DSC). The deuterium spectra arose from deuterons associated with the peptide through chemical exchange in ^2H_2O. The spectra from both peptides were characterized by a quadrupolar splitting parameter, $\omega_Q/2\pi \approx 150$ kHz, and an asymmetry parameter, $\eta \approx 0.17$. An additional 33 kHz, $\eta = 0$ component arising from deuterons on mobile ornithine side chains was present in gramicidin S. In the gel phase of dipalmitoylphosphatidylcholine liposomes the gramicidins gave spectra that had components identical with those obtained from the solids. In the liquid-crystalline phase gramicidin A containing samples gave multicomponent spectra with a maximum quadrupolar splitting value of 133 kHz, $\eta = 0$. A minimum in the T_{2e} was observed, coinciding with the onset of the broadened phase transition measured by DSC and ^{31}P NMR, due to the onset of axial rotation of the peptide in the bilayer. The different powder patterns in the liquid-crystalline spectra from gramicidin A probably arise from different amide sites along the transmembrane channel. The broad component of the ^2H NMR spectra from gramicidin S in liposome preparations was not affected by the lipid-phase transition. The T_{2e} was also constant over this temperature range. The results are consistent with a location of gramicidin S at the membrane surface.

The examination of lipid protein interactions in membranes is an active area of research. It has been stimulated by a large number of observations that indicate that the physical properties of membranes influence their function (McElhaney, 1982). Model membranes consisting of a few components have been used extensively for these studies to reduce the complexity of the interactions between membrane components and to allow systematic investigations to be made of the various types and strengths of these interactions. The use of model membranes also avoids the problems of instability and limited availability associated with membranes from natural sources. Various physical techniques especially electron spin resonance (ESR)[1] (Devaux, 1983) and NMR (Seelig & Seelig, 1980; Davis, 1983; Bloom & Smith, 1985) have been used in these investigations. The effects of proteins on the physical properties of the lipid matrix have been examined in some detail by these techniques, but the converse, i.e., the effects of the lipids on protein structure, has not received as much attention. Recent exceptions to this are studies of amino acid side-chain motion in several integral membrane proteins (Smith & Oldfield, 1984), a report of ^{15}N NMR spectra recorded from backbone sites of fd bacteriophage coat protein (Cross & Opella, 1982), and a ^2H NMR study of exchangeable sites on a synthetic polypeptide incorporated into DPPC bilayers (Pauls et al., 1985). The ^2H NMR study of the synthetic polypeptide in DPPC was the prototype for a class of experiments that can be carried out on membrane proteins to characterize their dynamical properties. Such experiments are based on the measurement of spectra arising from exchangeable hydrogen sites on polypeptide molecules in samples containing excess ^2H_2O. The ^2H_2O is necessary to avoid complications due to hydrogen exchange but makes the detection of the broad peptide spectrum difficult because it gives rise to an enormous solvent signal. A method for overcoming this solvent interference was published separately (Callaghan et al., 1984). In this paper we report the use of this type of experiment to examine the dynamical properties of gramicidin A and gramicidin S incorporated, separately, into liposomes prepared from DPPC.

An extensive body of literature indicates that the linear pentadecapeptide gramicidin A forms ion-permeable pores in natural or artificial membranes. Conductance studies (Hladky & Haydon, 1972; Andersen, 1983, 1984) and physical measurements (Urry, 1971; Urry et al., 1973, 1983; Wallace et

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[1] Abbreviations: ESR, electron spin resonance; NMR, nuclear magnetic resonance; FID, free induction decay; DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine; $[^2H]Me_2SO$, deuterated dimethyl sulfoxide; T_1, spin–lattice relaxation time; T_{2e}, spin–spin relaxation time; N_s, number of scans; T_R, time between repetition of pulse sequence; τ_2, time between 90° pulses of the quadrupolar echo sequence.

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al., 1981, 1982; Weinstein et al., 1979, 1980) have demonstrated that the functional form of the channel is a dimer consisting of two single gramicidin A helices joined N terminal to N terminal through six intermolecular hydrogen bonds. Low to medium concentrations of gramicidin A in lipid bilayers (below 1:4 molar ratio peptide to lipid) broaden their gel to liquid-crystal phase transition region (Chapman et al., 1977), increase the lipid chain order above T_c (Rice & Oldfield, 1979; Cortijo & Chapman, 1981), and decrease chain order below T_c (Cortijo & Chapman, 1981). In addition gramicidin A has a tendency to induce hexagonal H_{II} phase lipid organization in phosphatidylcholines with fatty acid chain lengths greater than 16 carbon atoms long (Van Echteld et al., 1982). The results of the present study show that changes in the phase behavior of membrane lipids influence the molecular motions of peptides such as gramicidin A that insert into the membrane matrix.

Gramicidin S is a circular decapeptide that has been shown to adopt a pleated sheet structure stabilized by four intramolecular hydrogen bonds in nonaqueous solvents (Stern et al., 1968; Ovchinnikov et al., 1970; Rae et al., 1977) and in the solid state (Hull et al., 1978; Huang et al., 1981; Krauss & Chan, 1982a,b). This antibiotic has been shown to disrupt and solubilize lecithin liposomes (Finer et al., 1969; Pache et al., 1972; Wu et al., 1978). Only a limited amount of work has been done to investigate its interaction with membrane molecules at a molecular level. The results to date indicate that the presence of charged groups in the second and seventh amino acid residues are essential for antimicrobial activity (Ovchinnikov & Ivanov, 1975). It has been suggested that the protonated amino groups of the ornithine side chain in these positions interact with the phosphate moiety of the lipid head groups (Finer et al., 1969; Pache et al., 1972).

Deuterium NMR spectra arising from exchangeable sites on the gramicidin A and S were obtained at various temperatures over a range that spanned the gel to liquid-crystalline phase transition temperature of the lipid bilayers. This was done to determine what effect this major reorganization in lipid order had on the peptide molecules associated with them.

Materials and Methods

Chemicals. Gramicidin A (actually a mixture of A, B, and C at a ratio of 7:1:2, respectively), sometimes called gramicidin D or A′ (Feigenson, 1983), was obtained from Boehringer Mannheim. Gramicidin S was obtained from Sigma Chemical Co., St. Louis. Both gramicidins were used without further purification. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0, 16:0 phosphatidylcholine) was obtained from Calbiochem or Sigma. [$^2H_3$]Ammonia gas (N^2H_3, 99 atom % ^2H), [$^2H_1$]methyl alcohol (CH_3O^2H, 99 atom % ^2H), sodium [$^2H_1$]hydroxide (NaO^2H, 99 atom % ^2H, 40% in ^2H_2O), deuterium oxide (^2H_2O, 99.7 atom % ^2H), and [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]Me_2SO, 99 atom % ^2H) were obtained from MSD Isotopes, Montreal.

Sample Preparation. Exchangeable sites on the gramicidins were deuterated by dissolving them in excess deuterated methanol (CH_3O^2H) at 40 °C. The exchange was catalyzed by adding a small amount of NaO^2H to the solution or alternatively by bubbling ammonia gas (N^2H_3) through it for approximately 5 min. Solvent was subsequently removed by evaporation overnight at reduced pressure.

Multilamellar lipid dispersions containing the gramicidins were prepared in ^2H_2O. Peptides and DPPC were codissolved in CH_3O^2H to give samples containing 15% gramicidin A or 20% gramicidin S by weight (13:1 and 6:1 lipid to peptide molar ratio, respectively). After the solvent was removed in vacuo, ^2H_2O was added to the solid mixture at a ratio of 1:1 (wt/wt %). Homogeneous membrane dispersions were obtained by mechanically mixing the samples after heating them to approximately 50 °C. Samples containing 1 g of DPPC were used for the ^2H NMR experiments. Portions of the same samples were used to obtain ^{31}P NMR spectra.

Proton Magnetic Resonance Spectroscopy. Spectra of undeuterated and deuterated (see Sample Preparation) gramicidin A in [$^2H_6$]Me_2SO were recorded at 400 MHz with a Bruker spectrometer using a 12-μs detection pulse.

Deuterium Magnetic Resonance Spectroscopy. ^2H NMR spectra were recorded at 35.46 MHz with a Nalorac superconducting magnet and a deuterium spectrometer constructed in the UBC Physics Department electronics shop. All spectra were taken on resonance by using the quadrupolar echo technique (Davis et al., 1976). Quadrature detection was used, and phases of the pulses in the quadrupolar echo sequence were adjusted to refocus all of the magnetization in the in-phase detection channel. As a consequence the out-of-phase channel, which contained only noise, could be zeroed. This results in an increase in the signal/noise ratio with $2^{1/2}$ and in the creation of an artificial perfectly symmetrical spectrum (Davis, 1983). To eliminate coherent noise and compensate for phase instabilities, the phases of the pulses making up the quadrupolar echo sequence were cycled, and the resulting positive and negative echoes were respectively added to or subtracted from the computer memory (Pauls et al., 1983).

The temperature of the sample was regulated by an oven that enclosed the radio-frequency coil. For temperatures lower than ambient nitrogen gas, evaporated from a liquid nitrogen containing Dewar, was directed through a channel in the oven casing to provide a heat sink. The first measurements (spectra and relaxation times) on all samples were taken at low temperatures, and after each temperature increase the samples were allowed to equilibrate for at least 0.5 h.

To remove the large solvent (^2H_2O) peak and extract the relatively weak signal arising from deuterons associated with the peptides, (1) a low order polynomial was fit to the slowly decaying portion of the FID arising primarily from the ^2H_2O, (2) the fit polynomial was extrapolated back to zero time, and (3) the extrapolated curve was subtracted from the whole. The Fourier transform of the signal obtained by this procedure corresponds to the peptide ^2H NMR powder spectrum (Callaghan et al., 1984).

Powder pattern deuterium spectra obtained from the samples were "DePaked" by a numerical procedure to give the equivalent oriented spectra (Bloom et al., 1981; Sternin et al., 1983). For powder spectra arising from nuclei with local axial symmetry (asymmetry parameter, $\eta = 0$) the DePaked spectrum is the true oriented spectrum. We use the convention of displaying the DePaked spectrum as though the external magnetic field was oriented parallel to the electric field gradient symmetry axis. Thus, a given ^2H site would produce a doublet with lines displaced by an angular frequency $\pm \omega_Q S$ with respect to the Larmor frequency. The splitting parameter ω_Q is related to the quadrupolar coupling constant e^2qQ/h, which is usually expressed in units of hertz, $\omega_Q = (3/4)e^2qQ/h$. The parameter S represents the reduction in the quadrupolar splitting due to motional averaging associated with rotation or conformational changes of the peptide.

For $\eta \neq 0$, the DePaked spectrum is not the true oriented spectrum but corresponds to that oriented spectrum arising from an axially symmetric ($\eta = 0$) site that would give the observed powder spectrum. As described by Sternin et al. (1983), the resulting DePaked spectrum extends over a range

of angular frequencies between

$$\omega(\pi/2) = \omega_Q S(1 + \eta) \quad (1)$$

which is the angular frequency for the *true* oriented spectrum for the external magnetic field oriented along the y axis of the electric field gradient principal axis coordinate system, and

$$\omega(0) = \omega_Q S(1 - \eta) \quad (2)$$

which corresponds to the x axis angular frequency. The quadrupolar coupling parameters are obtained from measurements of $\omega(\pi/2)$ and $\omega(0)$, as illustrated in Figure 3, and the following relations, which are obtained from eq 1 and 2:

$$\omega_Q S = \tfrac{1}{2}[\omega(\pi/2) + \omega(0)] \quad (3)$$

$$\eta = [\omega(\pi/2) - \omega(0)]/[\omega(\pi/2) + \omega(0)] \quad (4)$$

Spin–spin relaxation times (T_{2e}) were determined by varying the delay time, τ_2, between the 90° pulses of the quadrupolar echo. Typically a range of 40–200 μs was covered, and each measurement was the result of 1000 transients. The size of the quadrupolar echo (arising from the gramicidin) found on top of the slowly decaying ^2H_2O signal was measured at each τ_2 value. Spin–lattice relaxation times (T_1) were obtained by application of a 180° pulse for a variable time, τ_1, before the quadrupolar (detection) sequence. The pulse sequence was

$$(\pi/2)_y\text{–}\tau_2\text{–}(\pi/2)_x - \text{add echo} - \quad T_R \gg T_1$$

$$(\pi)\text{–}\tau_1\text{–}(\pi/2)_y\text{–}\tau_2\text{–}(\pi/2)_x - \text{subtract echo}$$

and the size of the refocused gramicidin signal in a FID of 1000 scans was measured as a function of delay time, τ_1, ranging from 0 to 200 ms.

Phosphorus Magnetic Resonance Spectroscopy. ^{31}P NMR spectra of the membrane samples containing gramicidin S or A that had been used for the ^2H NMR study were obtained with Bruker WP 200 Fourier transform spectrometers operating at 81.0 MHz. In the case of the DPPC sample plus gramicidin S, 500 FID's were accumulated by using a 7-μs 90° detection pulse with a repetition rate of 0.8 s. For the DPPC plus gramicidin A 5000 FID's were accumulated by using a 18-μs 90° detection pulse with a repetition rate of 0.9 s. All spectra were taken with a spectral width of 25 000 Hz in the presence of gated broad-band proton decoupling (>5 W). Exponential multiplication in the time domain corresponding to 50 Hz broadening in the frequency domain was applied to all spectra.

Differential Scanning Calorimetry (DSC). After the ^2H NMR measurement gramicidin S and A containing multilamellar lipid dispersions were diluted with ^2H_2O to concentrations suitable for DSC. Calorimetric heating curves for the gramicidin S or A containing DPPC vesicles were obtained with an MC 1 calorimeter (Microcal Inc., Amherst, MA). The different in heat capacity of the samples was measured against pure ^2H_2O using a heating rate of 20 °C/h.

Results

Gramicidin A. Deuterium labeling of the exchangeable sites on the gramicidin molecules was monitored by high-resolution proton NMR. The extent of labeling was determined by comparing the intensities of the amide proton resonances, occurring between 7.7 and 8.4 ppm and tryptophan indole NH sites at 10.7 ppm (Glickson et al., 1972; Heitz et al., 1979), in spectra from deuterated and nondeuterated gramicidin A dissolved in $[^2H_6]Me_2SO$. After the spectra from the two samples were normalized for peaks arising from nonexchangeable protons, it was calculated that 32% of the tryptophans were deuterated (Figure 1). The results indicated that the ratio of amide deuterons to tryptophan indole deuterons was 10.1:1; therefore, approximately 91% of the intensity in the ^2H NMR spectra obtained from gramicidin A arose from the amide deuterons.

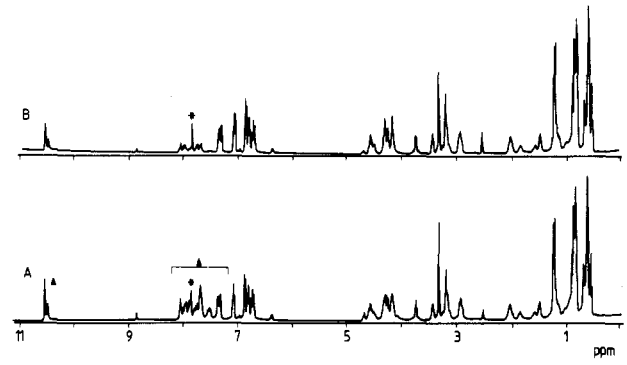

FIGURE 1: 400-MHz proton NMR spectra of (A) undeuterated gramicidin A and (B) deuterated gramicidin A in $[^2H_6]Me_2SO$ at room temperature. (▲) Exchangeable amide and tryptophan protons; (asterisk) nonexchangeable formyl proton.

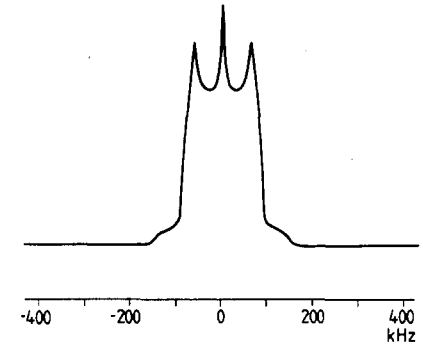

FIGURE 2: 35.46-MHz ^2H NMR spectrum of hydrogen-exchange deuterated gramicidin A in a solid powder at room temperature. N_s = 68 200, T_R = 1 s, and the time between 90° pulses of 1.45 μs in the quadrupolar echo sequence, τ_2 = 35 μs.

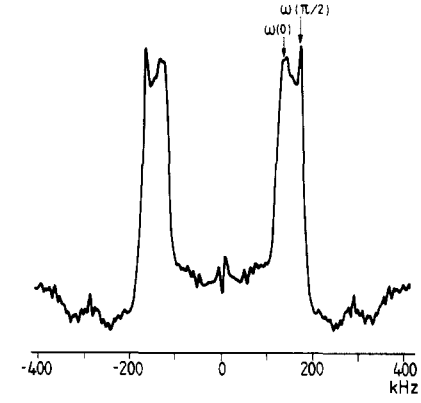

FIGURE 3: DePaked spectrum derived from the solid gramicidin A powder pattern of Figure 2. The left side shows the 8th iteration and the right side the 9th iteration.

Figure 2 shows the ^2H NMR spectrum of solid gramicidin A obtained from a powder of deuterated peptide at 23 °C. The spectrum has only one component approximately 150 kHz wide, with η = 0.18. The doublet as well as the negative intensities of twice the frequency, which are seen in the DePaked spectrum (Figure 3), are typical of a deuterium powder pattern with a nonzero asymmetry parameter (Sternin et al., 1983). Both T_1 and T_{2e} relaxation plots were single exponentials corresponding to relaxation times of 360 ms and 86 μs, respectively.

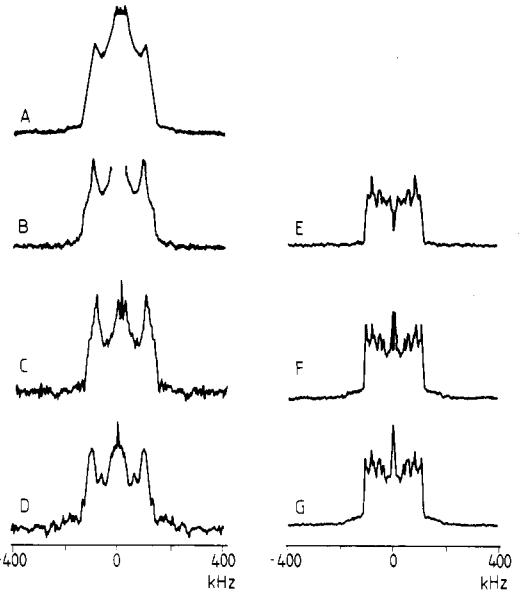

FIGURE 4: 35.46-MHz ^{2}H NMR spectra obtained at various temperatures from hydrogen-exchange deuterated gramicidin A incorporated into liposomes of DPPC (gramicidin A:DPPC = 1:13.0 molar ratio) prepared in ^2H_2O: (A) 12 °C, N_s = 325 000, T_R = 1 s, τ_2 = 40 μs; (B) 20 °C, N_s = 297 000, T_R = 200 ms, τ_2 = 35 μs; (C) 28 °C, N_s = 17 000, T_R = 1 s, τ_2 = 40 μs; (D) 36 °C, N_s = 69 000, T_R = 750 ms, τ_2 = 35 μs; (E) 44 °C, N_s = 240 998, T_R = 125 ms, τ_2 = 40 μs; (F) 48 °C, N_s = 723 000, T_R = 500 ms, τ_2 = 40 μs; (G) 52 °C, N_s = 525 000, τ_2 = 40 μs.

Figure 4 shows ^{2}H NMR spectra obtained at various temperatures from deuterated gramicidin A in DPPC bilayers. The spectra recorded at temperatures below 36 °C (Figure 4A–D) consist of a central peak (75 kHz at its base, at 4 °C) superimposed on a powder pattern with a quadrupolar splitting of approximately 150 kHz and an asymmetry parameter value of η = 0.17. In all of the ^{2}H NMR spectra obtained by the solvent subtraction technique (Callaghan et al., 1984) the central portion (approximately 5 kHz on each side of zero) is distorted by the procedure. The central peak becomes narrower as the temperature is increased and is not present in the spectra obtained above 37 °C (Figure 4E–G). Spectra obtained at 37 °C and higher consist of several powder patterns with different quadrupolar splitting values up to 133 kHz. The result of the "DePaking" procedure indicates that the asymmetry parameter value for these powder patterns is zero (Figure 5C,D). Increasing the temperature above 40 °C decreased the maximum splitting value by a few kilohertz and resulted in changes in the relative intensities of the peaks in the spectrum but did not change their number (Figure 5C,D).

The change in shape of the spectrum from the single nonzero asymmetry parameter value powder pattern to the multipeaked spectrum at 37 °C for the DPPC plus gramicidin A sample coincided with the peak in the DSC scan of the same preparation (Figure 6B). The phase transition in the DSC scan extended from approximately 34 to 46 °C and was also observed in ^{31}P NMR spectra obtained from the sample. All of the ^{31}P NMR spectra were typical bilayer spectra with a low-field shoulder and a high-field peak (Figure 7A–F). At 35 °C and below they were broad with a chemical shift anisotropy parameter value (evaluated from the separation of peak and shoulder resonance positions) of 66 ppm whereas those obtained above 35 °C had chemical shift anisotropy parameter values approximately 10 ppm less (Figure 7A–F). No evidence of a peak at −11 ppm indicating the presence of H_{II} phase lipid (Van Echteld et al., 1982) was seen at any temperature.

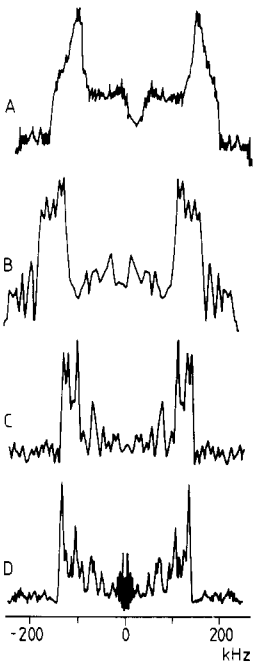

FIGURE 5: DePaked spectra derived from the powder patterns illustrated in Figure 4. The 9th (left) and 10th (right) iterations of the DePaking program are shown. (A) 12, (B) 28, (C) 44, and (D) 52 °C.

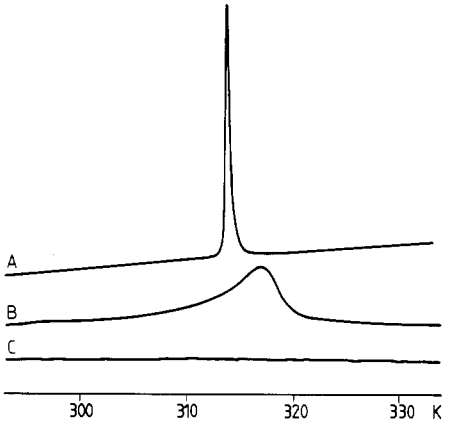

FIGURE 6: Differential scanning calorimeter traces of excess heat capacity for (A) DPPC liposomes plus gramicidin S (lipid to protein molar ratio = 6.0:1) in ^2H_2O. (B) DPPC liposomes plus gramicidin A (lipid to protein molar ratio = 13.0:1) in ^2H_2O, and (C) reference scan of ^2H_2O. The scan rate for all traces was 20 °C/h.

An Arrhenius plot of the ^{2}H NMR quadrupolar echo relaxation time (T_{2e}) at temperatures between 4 and 52 °C shows a minimum that coincides with the onset of the phase transition (Figure 8). Values for T_{2e} at 32 and 36 °C were approximately one-third of those observed at either 4 or 52 °C.

Gramicidin S. The ^{1}H NMR peak intensities of the gramicidin S amide hydrogens that occur between 7 and 9 ppm in $C^2H_3O^2H$ (Stern et al., 1968) were used to calculate the extent of deuteration. Addition of NaO^2H to the methanolic solution induced complete exchange of deuterium for protons within 1 h (data not shown).

The ^{2}H NMR spectrum of solid gramicidin S taken after deuteration is shown in Figure 9. It consists of an isotropic central peak (8 kHz at half-height), a powder pattern with $\omega_Q S/2\pi \approx 33$ kHz (η = 0), and an asymmetrical powder pattern with $\omega_Q S/2\pi \approx 150$ kHz (η = 0.17). In the DePaked spectrum the integrated intensity of the double peak centered about 150 kHz is approximately twice that observed at 33 kHz. (Figure 10). The quadrupolar echo decay for solid gramicidin

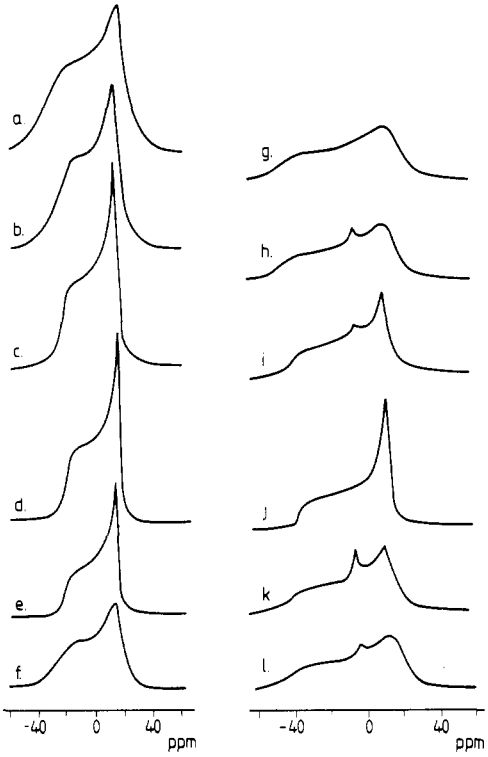

FIGURE 7: 81.0-MHz ^{31}P NMR spectra of the sample of the DPPC plus hydrogen-exchange deuterated gramicidin A liposome preparation used in Figure 4. Spectra were recorded sequentially at (a) 25, (b) 35, (c) 40, (d) 45, (e) 55, and (f) 30 °C; N_s = 5000, and T_R = 0.9 s. Proton decoupling > 5 W. 81.0-MHz ^{31}P NMR spectra of the sample of the DPPC plus hydrogen-exchange deuterated gramicidin S liposome preparation used in Figure 11. Spectra were recorded sequentially at (g) 25, (h) 35, (i) 40, (j) 45, (k) 37, and (l) 30 °C; N_s = 500, and T_R = 0.8 s. Proton decoupling > 5 W.

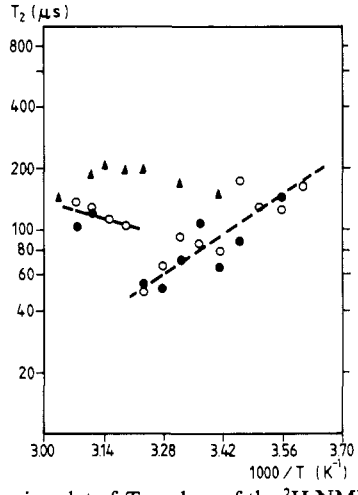

FIGURE 8: Arrhenius plot of T_{2e} values of the ^2H NMR quadrupolar echoes arising from deuterons exchanged onto gramicidin A and gramicidin S in gramicidin/DPPC/^2H_2O samples. The open and closed circles represent two different gramicidin A samples. The triangles represent the gramicidin S sample.

S was found to follow a single exponential with T_{2e} = 170 μs. The T_1 curve, in contrast, was complex. Three exponentials with time constants of 10, 50, and 180 ms were observed. The values were found to belong to the isotropic peak, the 33-kHz pattern, and the 150-kHz pattern, respectively, by measuring the relaxation in the frequency domain.

^2H NMR spectra obtained from multilamellar dispersions of DPPC plus deuterated gramicidin S at a protein to lipid molar ratio = 1:6.0 are shown in Figure 11. All of the spectra have a powder pattern with $\omega_Q S/\pi \approx 150$ kHz and a nonzero

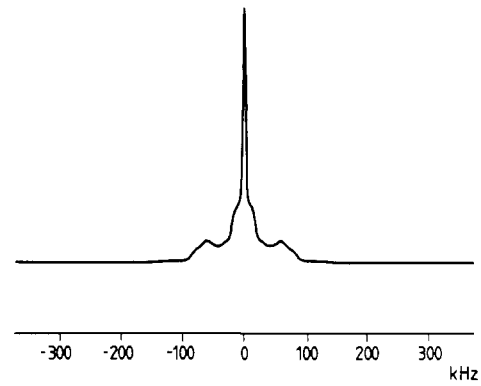

FIGURE 9: 35.46-MHz ^2H NMR spectrum of hydrogen-exchange deuterated gramicidin S in a solid powder at room temperature; N_s = 5000, T_R = 1 s, and τ_2 = 40 μs.

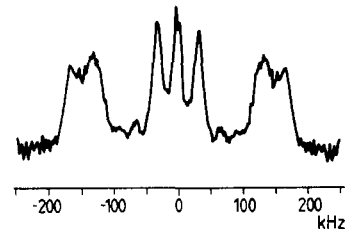

FIGURE 10: DePaked spectrum derived from the solid gramicidin S powder pattern of Figure 9. The left side shows the 8th iteration and the right side shows the 9th iteration.

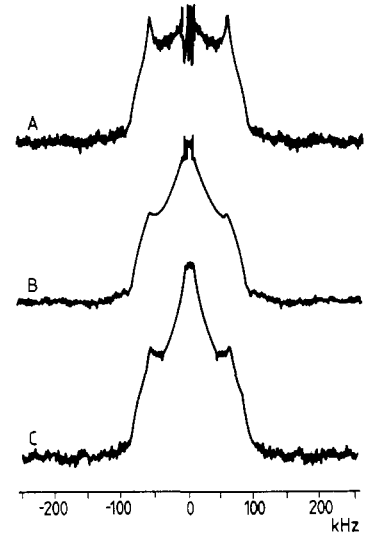

FIGURE 11: 35.46-MHz ^2H NMR spectra obtained at various temperatures from the hydrogen-exchange deuterated gramicidin S/DPPC/^2H_2O sample (proton to lipid molar ratio = 1:6.0). (A) 20 °C, N_s = 61 301, T_R = 1 s, τ_2 = 35 μs; (B) 36 °C, N_s = 111 000, T_R = 500 ms, τ_2 = 35 μs; (C) 48 °C, N_s = 124 050, T_R = 500 ms, τ_2 = 35 μs.

asymmetry parameter value. In addition, the spectra obtained at 24 °C and above also have a narrower component that is approximately 75 kHz wide at its base. The splitting of the broad component did not change over the temperature range studied (12–56 °C) even though the sample went through the gel to liquid-crystalline phase transition. DSC of the sample showed that the addition of gramicidin S at a protein to lipid ratio of 1:6.0 shifted the phase transition of DPPC down by approximately 1.5 °C to 40 °C and broadened the width slightly (Figure 6A). The transition was completed by 42 °C. The phase transition was also evident in the ^{31}P NMR spectra obtained from the gramicidin S containing sample. Parts G–L of Figure 7 show a decrease in the chemical shift anisotropy parameter value at 40 °C. In addition the ^{31}P NMR spectra

had an isotropic peak between 25 and 40 °C which included a maximum of 8% of the intensity at 37 °C and was not present in the liquid-crystalline spectra. The transition had no effect on the T_{2e} of the gramicidin S associated deuterons. The Arrhenius plot of this value (Figure 8) shows that T_{2e} remained approximately at 180 μs throughout the temperature range studied.

Discussion

The results of this study indicate that there are significant differences in the way gramicidin A and gramicidin S interact with DPPC bilayers. This is consistent with information obtained previously for these two antibiotic peptides produced by *Bacillus brevis*.

The ^{2}H NMR spectrum obtained from solid gramicidin A had a large quadrupolar splitting of $\omega_Q S/2\pi \approx 150$ kHz typical of rigid amide deuterons hydrogen bonded to oxygen atoms in a crystalline solid (Hunt & MacKay, 1976; Pauls et al., 1985). In fact the high-resolution proton NMR spectra obtained from the samples used in this study show that 91% of the deuterons exchanged onto gramicidin A were associated with the amide groups of the backbone. X-ray diffraction studies of gramicidin A crystals have indicated that the peptide assumes the form of a helical dimer in the solid that is stabilized by hydrogen bonds between the amide hydrogens and carbonyl oxygens of the backbone (Koeppe et al., 1979; Andersen, 1984). Quadrupolar splitting values for N–^{2}H–O groups have previously been shown to scale as the inverse of the cube of the hydrogen bond distance (Hunt & MacKay, 1976). Therefore, the observation that all of the deuterons associated with gramicidin A have the same quadrupolar splitting value indicates that there is little variation in the hydrogen bond distances along the peptide backbone, and we can assume, with confidence, that $S = 1$ and $\omega_Q/2\pi = 150$ kHz for this system.

The ^{2}H NMR spectrum obtained from gramicidin A in gel-phase bilayers of DPPC differed only slightly from that recorded from the solid. In particular the broad components of the two spectra were very similar with $\omega_Q S/2\pi = 145$ kHz and $\eta = 0.22$ in the gel phase compared to 150 kHz and 0.18 for the solid. This indicates that on the NMR time scale of 10^{-5} s the backbone deuterons of gramicidin A are almost as immobilized in a gel-phase bilayer as in the pure solid. The difference betwee the two preparations is the presence of a central component in the spectra from the membrane sample that is not present in the spectrum obtained from solid gramicidin A. The origin of this narrow spectrum was not determined. This component could be an artifact of the solvent subtraction technique used but previous experience with this procedure has indicated that spectral distortions are restricted to ±5 kHz about zero. Alternatively it may arise from monomeric forms of gramicidin A in the bilayer that may have more motional freedom than the dimer. This proposal is consistent with the disappearance of this narrow component at higher temperatures since the decrease in the thickness of the membrane that occurs during the gel to liquid-crystalline phase transition (Büldt et al., 1978) would favor gramicidin dimer formation. This is because the DPPC bilayer is slightly thicker than the length of the gramicidin A dimer, making it difficult for the intramolecular bonds involved in dimer formation to be formed, especially in the gel phase.

The broad spectral component arising from amide deuterons of gramicidin A in DPPC bilayers remained unchanged as long as the lipid was in the gel state. However, between 35 and 45 °C it was transformed from a single powder pattern with a nonzero asymmetry parameter to a multipeaked spectrum with asymmetry parameter values equal to zero. Both ^{31}P NMR and DSC (Papahadjopoulos et al., 1975) measurements indicated that the sample undergoes a gel to liquid-crystalline phase transition over this temperature range. The change in spectra shape suggests the onset of new motions in the gramicidin dimer during the lipid phase transition. Previous results from a synthetic amphiphilic polypeptide (Lys_2-Gly-Leu_{24}-Lys_2-Ala-amide) which forms an α-helix in DPPC bilayers indicated the same sort of behavior for the peptide at the lipid-phase transition. The synthetic peptide was found to be immobilized in gel-phase bilayers on the ^{2}H NMR time scale (10^{-5} s) but gave ^{2}H NMR spectra above the phase transition that corresponded to that expected from a peptide reorienting rapidly about the symmetry axis of the α-helix (Pauls et al., 1985). This interpretation of the spectral data was supported by quadrupolar echo relaxation time measurements for the deuterons associated with the peptide. They indicated the occurrence of a minimum value in T_{2e} at a temperature coincident with the phase transition of the bilayer. According to general NMR theory of relaxation (Abragam, 1961) discussed previously for the synthetic peptide (Pauls et al., 1985), a minimum in the T_{2e} value is predicted for systems undergoing motion that accelerate from a regime where their correlation times are long on the ^{2}H NMR time scale ($\gg 10^{-5}$ s) to one where their correlation times are much less than 10^{-5} s. The Arrhenius plot of T_{2e} for backbone deuterons on gramicidin A in DPPC, obtained in the present study, also had a minimum value at a temperature coincident with the onset of the lipid-phase transition. By analogy with the reasoning used previously to explain the synthetic peptide results, the minimum in the temperature dependence of T_{2e} for gramicidin A in DPPC may be associated with the onset of axial rotation of gramicidin A in the lipid bilayer. This is made possible by the occurrence of fluid-phase lipid at the onset of the lipid-phase transition which makes up an increasing proportion of the total lipid as the temperature of the sample is increased.

Also coinciding with the lipid-phase transition was a change in the spectrum, arising from the amide deuterons. Assuming fast axial rotation of the gramicidin A dimer and the long molecular axis of this dimer parallel to the bilayer normal, the quadrupolar splittings could reflect differences in the angle, θ, between the N–^{2}H vector along the backbone and the molecular axis of rotation. For liquid-crystalline spectra with $\eta = 0$, the quadrupolar splitting parameter becomes

$$\omega(\theta) = \omega_Q S_{N\text{-}^2H} = \omega_Q[\tfrac{1}{2}(3\cos^2\theta - 1)] \qquad (5)$$

If θ takes different values because of librational motions or changes in molecular conformation, this expression must be averaged over an appropriate distribution of θ. From the most prominent peaks in the spectra three values for $\omega(\theta)$ can be found: 133, 111, and 75 kHz. For any value $\omega(\theta) > 75$ kHz a unique solution for θ can be calculated by using eq 5. However, since we only measure the absolute value of $\omega(\theta)$, for any $\omega(\theta) \leq 75$ kHz, two solutions of θ are possible. Under the assumption of pure axial rotation, angles of 16° (133 kHz), 25° (111 kHz), and 35° or 90° (75 kHz) can be calculated. According to the model of Urry (1971) for the gramicidin A dimer [$\pi_{(LD)}$helix] three different amide sites can be identified, namely, non-hydrogen-bonded amide deuterons, amide deuterons in 14-membered hydrogen-bonded rings, and amide deuterons in 16-membered hydrogen-bonded rings. Given an intenstiy of approximately 1:2:3 in the DePaked spectra (Figure 5C,D) for the 75-, 111-, and 133-kHz splitting patterns, we suggest they arise from the nonbonded deuterons, the deuterons of the 14-membered hydrogen-bonded rings, and the deuterons of the 16-membered hydrogen-bonded rings,

respectively. This would be consistent with the $\pi_{(LD)}$ helix model for the gramicidin A dimer (Urry, 1971) since the non-hydrogen-bonded amides can be expected to have the least order because of motion at the ends, followed by the deuterons in the 14-membered rings, which make a greater angle to the molecular axis than the deuterons in the 16-membered rings.

It is interesting to compare the ^2H NMR results obtained from the gramicidin A dimer with those from a synthetic polypeptide, which forms an α-helix in DPPC bilayers. The latter gives a single powder pattern of 127 kHz and $\eta = 0$, indicating equivalent N–^2H bonds, all making an angle of 19° with respect to the bilayer normal. Thus, the ^2H NMR technique applied to exchangeable hydrogens of peptides may be a way to discriminate between models for their organization in membranes.

The broad component of the deuterium spectra obtained from solid deuterated gramicidin S was very similar to that observed for solid gramicidin A. As discussed previously the 150-kHz splitting indicates little motion on the ^2H NMR time scale of 10^{-5} s. This is consistent with other findings for gramicidin S which indicate that the pleated sheet structure is an extremely rigid conformation found in the crystalline form of gramicidin S (Hull et al., 1978) and is retained in solvents differing widely in their polarity (Ovchinnikov & Ivanov, 1975). Furthermore, the similarity of the spectra suggests that ^2H NMR spectroscopy of backbone deuterons in solid proteins cannot be used to distinguish between helical or pleated sheet structures. In fact, spectra obtained from a variety of peptides and proteins deuterated by chemial exchange that differ with respect to their secondary structure were all very similar (unpublished observations). This indicates that this technique is not sensitive to differences in protein secondary structure. In addition to the 150-kHz powder pattern, the spectra from solid gramicidin S also had a 33-kHz powder pattern that can be attributed to the rotating terminal $N^2H_3^+$ groups of the ornithine side chains (Hunt & MacKay, 1976). Theoretically the intensity ratios of the two components in the DePaked spectrum would be 8 to 6 for the amide to ornithine deuterons. However, the measured ratio is 9.5 to 4.5. A possible explanation for the extra intensity found at the 150 kHz is that part of the terminal $N^2H_3^+$ groups of the ornithine side chains are as immobile as the backbone deuterons.

The DePaked spectrum of solid gramicidin S shows that the asymmetry parameter for the 33-kHz component is equal to zero because it has one peak at this frequency (Sternin et al., 1983). Like gramicidin A, the spectra obtained from the gramicidin S/liposome preparation at temperatures below the lipid-phase transition did not differ from the solid spectrum. However, unlike gramicidin A, raising the temperature of the DPPC/gramicidin S mixture thorugh the lipid-phase transition had no effect on the 150-kHz splitting in the deuterium spectrum. Gramicidin S in DPPC bilayers at a protein to lipid ratio of 1:10 (compared to 1:6.0 as used in our experiments) was shown to be partially self-aggregated for approximately 45 ± 10% outside the bilayers (Wu et al., 1978). To examine what affect this nonassociated gramicidin would have on the ^2H NMR spectrum, a mixture of deuterated gramicidin S and ^2H_2O was examined by ^2H NMR. The spectrum obtained from this sample consisted of a narrow isotropic peak that could be removed by applying the solvent subtraction technique (data not shown). Therefore, a contribution from self-aggregated gramicidin S to the broad ^2H NMR spectrum observed in the gramicidin S/DPPC samples can be ruled out. The insensitivity of the powder line shape of the gramicidin S/liposome preparation to the large increase in thermal motion of the phospholipid side chains that occurs during the lipid phase transition suggests that the gramicidin S molecules associated with the DPPC bilayers are not in contact with the hydrophobic portion of the membrane. Consistent with this view is the constancy of the T_{2e} value over the phase transition temperature range. It is clear, however, from the DSC and ^{31}P NMR results that gramicidin S does interact with phospholipids; it does abolish the pretransition, shifts the main transition to a slightly lower temperature, and induces an isotropic peak in the ^{31}P NMR spectra between 25 and 40 °C. All of the observations support a previous proposal that gramicidin S interacts with phospholipid head groups. It has been suggested that the amino groups of the ornithine side chains form salt linkages with the phospholipid head groups (Finer et al., 1969; Pache et al., 1972).

The ^2H NMR results can best be explained by assuming that (1) at low temperatures the interfacially located gramicidin S is aggregated and (2) at 24 °C and above the increased lateral phospholipid mobility as well as the formation of isotropic particles also increases the mobility of a population of gramicidin S molecules. In this model the ornithine group would be immobilized and contribute to the broad component of the spectrum (as observed for the solid gramicidin S). Increased phospholipid mobility results in a reduction in the quadrupolar splitting of the amide bonds which accounts for the central component at temperatures above 24 °C.

In summary the use of ^2H NMR with peptides that have become labeled by chemical exchange gives information about the structure and dynamics of these molecules in the solid state and, after incorporation, also in artificial membrane systems. Two different patterns of association between lipids and proteins are modeled by gramicidin A and S. The former was found to be markedly sensitive to the increase in thermal motion of the phospholipid acyl chains consistent with the transmembrane nature of the channel that the peptide creates. The latter was not affected by the lipid-phase transition consistent with the surface location that has been proposed for the peptide.

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